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DIE VORAUSBERECHNUNG VON GASCHROMATOGRAPHISCHEN RETENTIONSZEITEN AUS STATISTISCH ERMITTELTEN "POLARITÄTEN"

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EINLEITUNG

Die gaschromatographische Trennung erfolgt bei der Gas-Flüssigkeitschromatographie unter Verwendung einer stationären Flüssigkeit, in der sich die Substanzen der Probe mit unterschiedlichem Verteilungskoeffizienten lösen. Die unterschiedliche Löslichkeit ist die Ursache für die verschiedenen Wanderungsgeschwindigkeiten der einzelnen Stoffe auf der Trennsäule.

Bei der Vielzahl der bereits beschriebenen und erhältlichen stationären Flüssigkeiten besteht ein ständig wachsendes Bedürfnis nach Ordnung dieser Stoffe entsprechend ihrem Trennverhalten. Noch immer wird in der überwiegenden Mehrzahl der Fälle die für eine analytische Trennung geeignete stationäre Flüssigkeit durch Probieren gefunden. Soll die Auswahl der besten Trennsäule jedoch rechnerisch aus irgendwelchen für die Trennflüssigkeit charakteristischen Daten erfolgen, so muss aus diesen Daten das Trennverhalten der Flüssigkeit ersichtlich sein. Eine Charakterisierung von Trennflüssigkeiten sollte deshalb die Vorausberechnung gaschromatographischer Retentionszeiten ermöglichen.

Das Problem der Berechnung gaschromatographischer Retentionszeiten ist eine Frage der Charakterisierung von Trennflüssigkeit und Gelöstem. Beide Stoffe müssen durch jeweils eine Anzahl Grössen derart beschrieben werden, dass aus der Kombination der Daten für die Trennflüssigkeit mit denen des Gelösten die Retentionszeit ermittelt werden kann.

Da die Retentionszeit in unmittelbarem Zusammenhang mit dem Aktivitätskoeffizienten des Gelösten in der stationären Flüssigkeit steht, ermöglicht die Vorausberechnung des Aktivitätskoeffizienten eine Voraussage der Retentionszeit bzw. des spezifischen Retentionsvolumens V_g:

Nach KHAN[1] ist:

$$V_g = \frac{273\,R}{M_L p^\circ \gamma^\infty_p}$$

wobei M_L = Molekulargewicht der stationären Flüssigkeit; p° = Gleichgewichtsdruck des reinen gelösten Stoffes bei der Kolonnentemperatur; γ^∞_p = Aktivitätskoeffizient des Gelösten bei unendlicher Verdünnung und R = Allgemeine Gaskonstante.

Aus der Summe der Anteile von sechs Wechselwirkungen zwischen den je-

weiligen funktionellen Gruppen und Kohlenwasserstoffresten eines Zweistoffgemisches ermittelten PIEROTTI und Mitarb.[2] die Aktivitätskoeffizienten bei unendlicher Verdünnung von einem umfangreichen Material. Ausser der C-Zahl von Trennflüssigkeit und Gelöstem benutzt PIEROTTI bis zu 7 Konstanten, aus denen sich Aktivitätskoeffizienten mit guter Genauigkeit vorausberechnen lassen.

Nach MATIRE[3] lässt sich der Aktivitätskoeffizient nach einem Ansatz von VAN LAAR, HILDEBRAND u.a. berechnen, wenn von der Trennflüssigkeit das Molvolumen, die Dielektrizitätskonstante, der Siedepunkt und ein empirischer Korrekturfaktor bekannt sind. Die gleichen Grössen, mit Ausnahme des Korrekturfaktors werden für das Gelöste benötigt. MATIRE berechnete die Retentionszeiten für Cyclohexan, Benzol und 2,4-Dimethylpentan auf Dimethylformamid mit einer Genauigkeit von 1–6 %. Ob diese Berechnungsmethode auch für andere Trennsäulen, namentlich für solche ohne definierten Siedepunkt bzw. ohne einheitliches Molekulargewicht und für andere Substrate, z.B. für Stoffe mit Dipolmoment anwendbar ist, muss noch geprüft werden.

Zur Berechnung von Retentionszeiten aus dem Aktivitätskoeffizienten wird ausserdem das Molekulargewicht der Trennflüssigkeit und der Dampfdruck des Gelösten bei der Analysentemperatur benötigt. Alle diese Zahlen sind oft nur schwer zugänglich, so dass die Vorausberechnung von Retentionszeiten aus dem Aktivitätskoeffizienten bisher wegen der zahlreichen und oft nicht bestimmten Konstanten der beiden Komponenten praktisch nicht durchgeführt wurde.

Die Voraussage *relativer* Retentionszeiten aus empirisch gefundenen Zusammenhängen hat grössere praktische Bedeutung erlangt. Einerseits ist der Analytiker immer nur an dem Verhältnis zweier Retentionszeiten interessiert, andererseits ist zu erwarten, dass ein Teil der für die Berechnung des Aktivitätskoeffizienten notwendigen Kräfte für die Ermittlung relativer Retentionszeiten unberücksichtigt bleiben kann, da diese Kräfte auf die beiden gelösten Stoffe gleichartig wirken.

Siedepunkt und Logarithmus der Retentionszeit stehen in annähernd linearem Zusammenhang. TENNEY[4] untersuchte die Selektivität stationärer Flüssigkeiten, indem er die auf Pentan bezogenen relativen Retentionszeiten homologer Reihen bestimmte und gegen den Siedepunkt auftrug. Auf diese Art lässt sich die Trennflüssigkeit mit der höchsten Selektivität zwischen gleichsiedenden Stoffen zweier homologer Reihen ermitteln. BAYER[5] definierte einen Selektivitätskoeffizienten, der ein Mass für diese Selektivität darstellt. Vorausberechnungen von Retentionszeiten nach dem Selektivitätskoeffizienten sind kaum möglich, die vollständige Charakterisierung einer Trennflüssigkeit durch Selektivitätskoeffizienten erfordert die Angabe der Selektivitäten zahlreicher homologer Reihen.

Mit sehr guter Genauigkeit lässt sich nach KOVÁTS[6] die Differenz der auf die Alkane bezogenen Retentionszeiten von zwei Trennsäulen berechnen. Dabei wird angegeben, um welchen Betrag sich ein Stoff im Vergleich zu einem Paraffin auf einer polaren Trennsäule anders verhält als auf einer unpolaren. KOVÁTS schlägt zur Charakterisierung einer Trennflüssigkeit die Angabe von Inkrementen vor, die für homologe Reihen charakteristisch sind.

Ähnliche Vorschläge stammen von EVANS[7] sowie von MERRITT UND WALSH[8], die gleichfalls für homologe Reihen charakteristische Grössen zur Kennzeichnung von Trennflüssigkeiten angeben. Diese Daten haben den Nachteil, für die ersten zwei bis vier Glieder der homologen Reihen, die bei der praktischen Arbeit häufig auf-

treten, unzureichende Werte zu liefern und eine beträchtliche Anzahl von Angaben — für jede homologe Reihe eine — für die Beschreibung einer Trennsäule zu erfordern.

Bei unserer Untersuchung der Polarität stationärer Flüssigkeiten[9] stellten wir fest, dass sich zwar Paraffine, Olefine und Diolefine in ihrem Retentionsverhalten auf verschiedenen Trennsäulen durch die Angabe einer "Polarität" hinreichend genau beschreiben lassen, die Laufzeiten von Acetylen aber sich nicht in dieses System einfügten.

BROWN[10] fordert zur Beschreibung von Trennflüssigkeit oder Gelöstem die Angabe von je drei Grössen: Eine Gesamtpolarität, sowie die Stärke der Elektronendonator- und -acceptorwirkung. BROWN charakterisiert eine Trennflüssigkeit nach ihrer Lage in einem Dreieckskoordinatensystem je nach den Anteilen der Laufzeiten eines unpolaren Stoffes, eines Elektronendonators oder -acceptors an der Summe aller drei Zeiten. Diese Charakterisierung erlaubt keine Vorausberechnung von Retentionszeiten und ist abhängig von der Wahl der drei Bezugssubstanzen.

Nach dem bisherigen Stand ist es z.B. nicht möglich, für die Praxis ausreichende Voraussagen über die Laufzeit von Äthanol auf einer Polyester- oder Silikonölsäule zu machen, da sich Äthanol einerseits nicht wie ein normales Glied der homologen Reihe der primären *n*-Alkohole verhält, andererseits zur Berechnung des Aktivitätskoeffizienten die notwendigen Angaben für Polyester oder Silikonöl fehlen.

Ziel dieser Untersuchung war es, eine Trennflüssigkeit durch möglichst wenige Daten so zu charakterisieren, dass eine für die Praxis ausreichend genaue Voraussage gaschromatographischer Retentionszeiten möglich ist.

BESTIMMUNG DER RETENTION

Die Retentionszeiten bei 100° wurden für die erste Versuchsserie (40 Stoffe auf 10 Trennsäulen) auf einem Fraktometer 116 E der Firma Perkin-Elmer mit Hitzdrahtdetektor bestimmt, die der zweiten Serie (15 Stoffe auf 21 Säulen) mit einem Fraktometer F 6/2 H der gleichen Firma. Trägergas war in beiden Fällen Wasserstoff, die Beladung des Trägers mit stationärer Flüssigkeit betrug in allen Fällen 20 Gew. %. Die verwendeten stationären Flüssigkeiten und die Säulenlängen sind aus Tabelle I zu entnehmen. Bei einigen unpolaren Trennflüssigkeiten (Squalan, Silikonöl, Fluorosilikon) war es notwendig, 0.5 % Alkaterg T der flüssigen Phase zuzusetzen, um die Schwanzbildung (tailing) bei polaren Stoffen zu verringern. Die Strömungsgeschwindigkeiten wurden so eingestellt, dass die zuletzt austretenden Substanzen eine Laufzeit von 30–45 Min aufwiesen.

Aus dem Vergleich der gemessenen Retentionszeiten mit den Laufzeiten der *n*-Alkane berechneten wir für jeden Stoff den Retentionsindex I nach KOVÁTS[6]. Dabei ist die Retentionszeit eines Stoffes durch seine Lage zwischen den beiden benachbarten *n*-Paraffinen charakterisiert. Ein Retentionsindex von 650 bedeutet z.B., dass der Logarithmus der Retentionszeit dieses Stoffes genau zwischen den entsprechenden Werten für Hexan und Heptan liegt, wobei der Retentionsindex der *n*-Paraffine definiert ist als das 100-fache der C-Zahl des Moleküls (Hexan = 600). Der Abstand zwischen zwei benachbarten *n*-Paraffinen beträgt 100 Einheiten.

Die mittlere Differenz von 60 Doppelbestimmungen betrug auf zwei verschiedenen Fraktometern in grossem zeitlichen Abstand für 12 Stoffe auf 5 Trennsäulen 3.9 Einheiten.

TABELLE 1

TRENNSÄULEN

Nr.	Länge (m)	Stationäre Flüssigkeit	Lieferfirma*	I_{Benzol}	$I_{Äthanol}$	M_{Benzol}	$M_{Äthanol}$	$\frac{M_{Äthanol}}{M_{Benzol}}$
1	2	Squalan	Merck	648	383	0	0	—
2	2	Dioktylsebacat	Merck	722	547	74	164	2.22
3	2	Methyl-Phenyl-Silikonöl	Merck	726	490	78	107	1.37
4	2	Dinonylphthalat	Merck	734	568	86	185	2.15
5	2	Polypropylene Glycol	F&M	799	720	151	337	2.23
6	2	Polypropylenglykol 600	CWH	828	793	180	410	2.28
7	2	Trikresylphosphat	Merck	824	698	176	315	1.79
8	2	Polypropylene Sebacate	G&G	841	721	193	338	1.75
9	2	Polyäthylenglykol 1000	CWH	987	977	339	594	1.75
10	2	Äthylenglykol-bis-cyanäthyl-äther	Merck	1164	1143	516	760	1.47
11	2	Squalan	Merck	649	384	0	0	—
12	2	Methyl-Phenyl-Silikonöl	Merck	718	500	69	116	1.68
13	2	Dioktylsebacat	Merck	722	547	73	163	2.23
14	2	Silikonöl DC 710	DC	753	541	104	157	1.51
15	5	Fluoro-Silikon-QF-1	F&M	761	574	112	190	1.70
16	2	Polypropylene Glycol	G&G	781	693	132	309	2.34
17	2	Acetyl Tributyl Citrate	F&M	787	655	138	271	1.96
18	2	Trikresylphosphat	Merck	821	702	172	318	1.85
19	2	Marlophen 87	CWH	827	755	178	371	2.08
20	2	Polypropylene Sebacate	G&G	843	728	194	344	1.77
21	2	Marlophen 814	CWH	873	830	224	446	1.99
22	2	Tween 80	F&M	890	851	241	467	1.94
23	2	Carbowax 4000 Dioleat	F&M	911	851	262	467	1.78
24	5	Silicone Fluid Nitrile XF 1150	F&M	935	860	286	476	1.66
25	2	Carbowax 20 M	F&M	974	924	325	540	1.66
26	2	Carbowax 4000	F&M	975	934	326	550	1.69
27	2	Polyäthylenglykol 1000	CWH	986	977	337	593	1.76
28	2	Reoplex 400	Merck	1006	965	357	581	1.63
29	5	Diäthylenglykolsuccinat	F&M	1135	1135	486	751	1.55
30	2	Äthylenglykol-bis-cyanäthyl-äther	CWH	1188	1162	539	778	1.44
31	2	1,2,3-Tris-2-cyanäthoxy-propan	F&M	1247	1258	598	874	1.46

* Lieferfirmen: E. Merck A.G., Darmstadt; F & M Scientific Corporation, Avondale, Pa.; G & G = Griffin & George Ltd.; CWH = Chemische Werke Hüls A.G., 437 Marl; DC = Dow Corning.

POLARITÄT DER TRENNSÄULE

Die niedrigsten relativen Retentionszeiten wurden für alle Stoffe auf der unpolaren stationären Flüssigkeit Squalan erhalten. Eine polare Trennsäule hält einen polaren Stoff im Vergleich zu einem unpolaren länger fest als eine unpolare Säule, da zusätzliche Wechselwirkungskräfte (z.B. Dipol-Dipol-Assoziationen oder Induktionskräfte) die relative Retentionszeit eines polaren Stoffes erhöhen. Die Differenz zwischen den Retentionsindices eines Stoffes auf einer polaren und einer unpolaren Trennflüssigkeit ist proportional der Polarität der Säule (x) und der Polarität des Stoffes (a).

$$\Delta I = I_p - I_o = a \cdot x = M \qquad (1)$$

wobei I_p = Retentionsindex auf polarer Säule und I_o = Retentionsindex auf unpolarer Säule (Squalan).

Für einen unpolaren Stoff (a sehr klein), z.B. für einen Kohlenwasserstoff, ist auch auf einer sehr polaren Trennsäule ΔI sehr klein.

Misst man den Retentionsindex für einen Stoff auf mehreren Säulen (a = const.), so ist M proportional x und ein Mass für die Polarität der Säule gegenüber dem jeweiligen Stoff. Die Werte für M von Benzol und Äthanol auf sämtlichen untersuchten stationären Flüssigkeiten, sowie ihr jeweiliges Verhältnis sind in Tabelle I zusammengestellt.

Aus diesen Werten ergibt sich, dass die Polarität einer Trennsäule gegenüber Benzol anders ist als die z.B. gegenüber Äthanol. Das Verhältnis der M-Werte zweier Stoffe ist nicht konstant, eine Trennsäule lässt sich durch die Angabe *einer* Polarität nicht beschreiben. In Tabelle II sind die Differenzen zwischen den Retentionsindices auf zwei Trennsäulen, Polypropylenglykol (Säule 6) und Trikresylphosphat (Säule 7) angegeben. Man sieht, dass beide Säulen für zahlreiche Stoffe, z.B. die Aromaten, Cyclohexan, Aldehyde, den Dibutyläther oder Tetrachlorkohlenstoff die gleichen Retentionsindices haben, für diese Substanzen die Polaritäten beider Säulen also gleich sind. Sämtliche Alkohole dagegen und einige Chlorkohlenwasserstoffe werden auf Trikresylphosphat länger festgehalten. Diese stationäre Flüssigkeit muss also im Gegensatz zum Polypropylenglykol eine zusätzliche zwischenmolekulare Bindung mit bestimmten Stoffen eingehen können.

TABELLE II

DIFFERENZEN ZWISCHEN DEN RETENTIONSINDICES AUF DEN SÄULEN POLYPROPYLENGLYKOL (NO. 6) UND TRIKRESYLPHOSPHAT (NO. 7)

Benzol	4	Butyraldehyd	2
Toluol	— 2	Crotonaldehyd	— 3
Äthylbenzol	— 4	Tetrachlorkohlenstoff	— 2
Cumol	—14	Chloroform	39
Styrol	— 3	Methylenchlorid	43
Cyclohexan	2	Äthanol	95
Methylcyclohexan	— 3	Propanol	91
2-Äthylhexan	3	*n*-Butanol	89
2-Äthylhexen-1	1	Pentanol-1	84
Aceton	11	Cyclopentanol	72
Methyläthylketon	5	Isopropanol	87
n-Butylacetat	23	Allylalkohol	103
Di-*n*-butyläther	— 1	*tert.*-Butanol	58

ZWEITEILIGE POLARITÄTEN

Da eine Kennzahl (x in Gleichung 1) zur Beschreibung der Polarität einer Trennsäule nicht ausreicht, fügten wir zur Gleichung (1) ein additives Glied ($b \cdot x$) hinzu:

$$M = ax + by \qquad (2)$$

wobei b und y einen zusätzlichen Polaritätsfaktor für das Gelöste und die Trennflüssigkeit darstellen.

Für das System von zwei Säulen und zwei Stoffen ergeben sich damit vier gemessene M'-Werte und 8 unbekannte Polaritätsfaktoren (Tabelle III).

TABELLE III

SCHEMA DER RECHENGRÖSSEN

Stoff		*Säule I*		*Säule II*	
	Polarität	x_I	y_I	x_{II}	y_{II}
A	$\frac{a_A}{b_A}$	M_{IA}		M_{IIA}	
B	$\frac{a_B}{b_B}$	M_{IB}		M_{IIB}	

Dementsprechend gibt es vier Gleichungen mit 8 Unbekannten:

$$M_{IA} = a_A x_I + b_A y_I$$
$$M_{IIA} = a_A x_{II} + b_A y_{II}$$
$$M_{IB} = a_B x_I + b_B y_I$$
$$M_{IIB} = a_B x_{II} + b_B y_{II}$$

Dieses Gleichungssystem ist vierfach unterbestimmt. Es ist also möglich, vier Faktoren (z.B. a_A, x_{II}, b_B, J_{II}) zu wählen und die vier anderen dann aus den Retentionszeiten zu berechnen. Liegen so die Faktoren von zwei Stoffen fest, so lassen sich aus den Retentionszeiten dieser beiden Stoffe auf anderen Säulen die Polaritätsfaktoren der Säulen berechnen.:

$$x_I = \frac{\frac{b_B}{b_A} M_{IA} - M_{IB}}{a_A \frac{b_B}{b_A} - a_B} \qquad y_I = \frac{\frac{a_B}{a_A} M_{IA} - M_{IB}}{b_A \frac{a_B}{a_A} - b_B}$$

Entsprechend werden die Polaritätsfaktoren aller Stoffe aus ihrer Laufzeit auf zwei Trennsäulen bekannter Polarität ermittelt, so dass von den 400 gemessenen Retentionszeiten 2 mal 10 (für zwei Stoffe auf 10 Trennsäulen) und 38 mal 3 (38 Stoffe auf Squalan und zwei beliebigen Trennflüssigkeiten), insgesamt also 134 exakt berechenbar sind. Die anderen Zeiten sind dann durch die ermittelten Polaritäten festgelegt und stimmen mehr oder weniger mit den gemessenen Werten überein. Durch Anwendung der Fehlerausgleichsrechnung ist es möglich in schrittweiser Näherung mit dem gesamten experimentellen Material aus den Säulenpolaritäten die Stoffpolaritäten und umgekehrt so lange zu mitteln, bis die Summe der Quadrate von den Differenzen zwischen gemessenem und berechnetem M-Wert ein Minimum erreicht.

FEHLERAUSGLEICHSRECHNUNG

Die Differenz zwischen dem gemessenen M' und dem berechneten M beträgt für einen Stoff auf einer Säule:

$$M' - ax - by.$$

Für einen Stoff auf allen Säulen ist:

$$a = \frac{\sum M'}{\sum x} - b\frac{\sum y}{\sum x} \tag{3}$$

Die Summe der Fehlerquadrate beträgt dann für einen Stoff auf allen Säulen:

$$E = \sum\left(M' - \frac{x\sum M'}{\sum x} + \frac{xb\sum y}{\sum x} - by\right)^2$$

Soll E ein Minimum erreichen, so ist nach den Regeln der Fehlerrechnung (DAVIES[11]):

$$\frac{dE}{db} = 0$$

Die Lösung für b lautet:

$$b = \frac{-\frac{\sum y}{\sum x}\sum(x\cdot M') + \sum(yM') + \frac{\sum y}{\sum x}\cdot\frac{\sum M'}{\sum x}\sum x^2 - \frac{\sum M'}{\sum x}\sum(xy)}{\left(\frac{\sum y}{\sum x}\right)^2\sum x^2 - 2\frac{\sum y}{\sum x}\sum xy + \sum y^2} \tag{4}$$

Der Wert von a ergibt sich dann durch Einsetzen des Ergebnisses für b in Gleichung (3).

a und b werden so aus den vorhandenen x,y-Werten und den gemessenen M-Werten für jeden Stoff berechnet. Dann wird in Gleichung (4) und (3) x durch a und y durch b ersetzt und für jede Säule y und dann x errechnet, dann wieder a und b für jeden Stoff und so fort, bis E ein Minimum erreicht.

Die mittlere Differenz F_m zwischen dem gemessenen und berechneten Wert von M betrug für die bei der ersten Versuchsserie untersuchten 40 Stoffe auf 10 Trennsäulen 7.8 Einheiten nach KOVÁTS.

DER DRITTE POLARITÄTSFAKTOR

In einer zweiten Versuchsserie wurden die Retentionsindices von 13 Stoffen, die bereits in der ersten Serie enthalten waren, sowie von Dioxan und Phenylacetylen auf 21 verschiedenen Trennsäulen ermittelt. Die Polaritätsfaktoren (x,y) der Säulen wurden entsprechend Gleichung (4) und (3) bestimmt und hiermit erneut die Werte für a und b von den 13 Stoffen jetzt über alle 29 Säulen ermittelt. Die Differenzen zwischen den gemessenen und den berechneten Retentionszeiten betrugen für die stationären Flüssigkeiten Fluorosilikonöl und die verschiedenen Carbowaxtypen bis zu 76 Einheiten nach KOVÁTS. Diese noch vorhandenen Differenzen unterliegen einer Gesetzmässigkeit. Einer grossen negativen Differenz auf der Fluorsilikonsäule entsprach stets eine geringere positive auf den Polyäthylenglykolsäulen und umgekehrt. Es war daher möglich, durch Einführung eines dritten Polaritätsgliedes $(c\cdot z)$ den noch verbliebenen Fehler in der Vorausberechnung erheblich zu senken.

Den Wert von z_1 erhielten wir für jede Säule durch Addition der absoluten Differenzen der Stoffe Methyläthylketon, Crotonaldehyd, *n*-Butylacetat, Tetrachlorkohlenstoff, Chloroform, Benzol, Styrol und Cyclohexan und anschliessende Division der Summen durch 10. Die Grösse c wurde für jeden Stoff gleichfalls durch Addition der absoluten Fehler auf allen Säulen und anschliessende Division durch den zehnten Teil der Summe der Fehler aller 13 Stoffe auf den 29 Säulen ermittelt. Das Vorzeichen für c und z wurde so ausgewählt, dass das Produkt dieser Zahlen dem Vorzeichen der jeweiligen Abweichung entsprach. Differenzen zwischen dem gemessenen und berechneten Wert, die ein anderes Vorzeichen hatten, als dem Produkt von $c \cdot z$ entsprach, mussten bei der Summenbildung abgezogen werden.

Für die Alkohole Äthanol, Allylalkohol und Cyclopentanol, sowie für Dibutyläther und 2-Äthylhexen war keine Abhängigkeit des verbliebenen Fehlers vom dritten Polaritätsfaktor z_1 festzustellen. Für diese Stoffe beträgt also $c = 0$.

Auf dem gleichen Wege wurde auch für die erste Versuchsserie c und z_2 für 16 Stoffe und 9 stationäre Flüssigkeiten ermittelt. Da sich hierbei Werte für z_2 auf den Säulen 2–10 ergaben, die von den zuerst bestimmten z_1 abwichen, mussten die a, b- und c-Werte der Stoffe, die nicht in der zweiten Versuchsserie enthalten waren,

TABELLE IV a

POLARITÄTSFAKTOREN DER TRENNSÄULEN

Lfd. Nr.	*Stationäre Flüssigkeit*	*x*	*y*	*z*	F_m	*Zahl der Stoffe*
1	Squalan	0	0	0		40
2	Dioctylsebacat	1.51	2.53	6.0	5.5	40
3	Methyl-Phenyl-Silikonöl	3.64	1.28	5.2	6.4	40
4	Dinonylphthalat	2.92	2.52	10.6	3.1	40
5	Polypropylene Glycol	3.38	4.79	12.4	3.6	40
6	Polypropylenglykol 600	3.51	5.88	12.4	6.1	40
7	Trikresylphosphat	5.61	4.23	4.1	5.3	40
8	Polypropylene Sebacate	5.32	4.72	— 0.8	4.4	40
9	Polyäthylenglykol 1000	8.89	8.03	—14.5	6.7	40
10	Äthylenglykol-bis-cyanäthyläther	18.53	9.27	— 6.8	5.3	40
11	Squalan	0	0	0		13
12	Methyl-Phenyl-Silikonöl	3.35	1.38	6.0	7.0	13
13	Dioctylsebacat	1.46	2.45	5.5	3.7	13
14	Silikonöl DC 710	4.78	1.78	2.8	7.5	13
15	Fluoro-Silikon QF-1	8.22	1.46	38.1	10.7	13
16	Polypropylene Glycol	2.37	4.52	10.1	6.8	13
17	Acetyl Tributyl Citrate	4.08	3.65	9.5	5.6	13
18	Trikresylphosphat	5.48	4.27	3.8	5.2	13
19	Marlophen 87	4.13	5.22	6.9	4.8	13
20	Polypropylene Sebacate	5.44	4.70	— 1.5	2.3	13
21	Marlophen 814	5.01	6.16	— 0.7	6.8	13
22	Tween 80	5.58	6.53	0	5.9	13
23	Carbowax 4000 Dioleat	6.38	6.62	—10.0	5.0	13
24	Silicone Fluid Nitrile XF 1150	10.22	5.98	11.5	9.9	13
25	Carbowax 20 M	8.40	7.55	—17.9	7.4	13
26	Carbowax 4000	8.53	7.73	—16.2	6.2	13
27	Polyäthylenglykol 1000	8.59	8.30	—12.7	3.6	13
28	Reoplex 400	10.40	7.79	—15.5	4.4	13
29	Diäthylenglykolsuccinat	15.19	9.73	—20.1	7.6	13
30	Äthylenglykol-bis-cyanäthyläther	18.92	9.44	—12.5	6.9	13
31	1,2,3-Tris-2-cyanäthoxypropan	20.47	10.58	—12.0	8.7	13

TABELLE IV b

FAKTOREN DER STOFFE

	I_0	a	b	c	F_m	*Zahl der Säulen*
Benzol	649	16.11	22.36	—1.34	5.5	29
Toluol	757	16.68	20.77	—1.04	4.3	9
Äthylbenzol	847	16.17	21.51	—1.21	4.5	9
Cumol	907	16.06	19.80	—1.11	3.7	9
Styrol	873	18.69	29.79	—1.50	5.5	29
Cyclohexan	674	4.23	4.89	—0.71	5.8	29
Methylcyclohexan	737	2.82	4.19	—0.78	3.1	9
2-Äthylhexan	774	0.39	— 0.62	0	2.9	9
2-Äthylhexen-1	776	3.78	5.35	0	4.1	29
2,3-Dimethylpentan	675	1.66	— 1.33	0	4.9	9
Aceton	418	25.38	29.51	1.64	3.9	9
Methyläthylketon	531	24.71	27.26	1.46	6.0	29
n-Butylacetat	744	20.29	24.57	1.10	5.9	29
n-Dibutyläther	856	8.02	7.62	0	7.4	29
Acetaldehyd	332	19.61	30.14	0	3.9	9
Propionaldehyd	437	22.05	25.87	1.08	3.8	9
Butyraldehyd	537	22.76	24.17	1.27	2.8	9
Crotonaldehyd	580	26.39	37.43	1.63	7.1	29
Chlormethyl	327	9.50	23.39	0	6.9	9
Methylenchlorid	492	10.06	43.12	—1.01	8.7	9
Chloroform	588	6.21	47.82	—0.82	8.4	29
Tetrachlorkohlenstoff	656	7.38	20.66	—1.26	6.8	29
Methanol	307	8.16	70.02	0	7.6	9
Äthanol	384	10.40	61.50	0	7.7	29
Propanol	486	9.20	63.88	0	2.8	9
Butanol	593	9.22	64.62	0	2.9	9
Pentanol	693	9.26	66.12	0	4.3	9
Cyclopentanol	720	13.30	65.50	0	6.6	29
Isopropanol	433	9.15	56.30	0	4.1	9
Isobutanol	553	7.90	64.47	0	4.2	9
sec.-Butanol	542	9.90	54.36	0	3.2	9
tert.-Butanol	470	9.80	48.21	0	5.9	9
Pentanol-2	640	9.56	55.28	0	4.1	9
Pentanol-3	647	10.29	51.63	0	3.9	9
2-Methylbutanol-1	669	8.06	65.28	0	7.6	9
2-Methylbutanol-2	593	10.73	45.92	0	6.2	9
3-Methylbutanol-2	629	10.09	51.63	0	4.0	9
2,2-Dimethylpropanol-1	606	7.38	60.34	0	5.0	9
3-Methylbutanol-1	664	8.47	65.29	0	3.4	9
Allylalkohol	469	10.57	72.55	0	5.5	29

auf die zuerst bestimmten Polaritätsfaktoren (x, y, z_1) angepasst werden. Die neuen Werte von a, b und c ergaben sich für jeden Stoff durch die Lösung von je drei Gleichungen:

$$M = ax + by + cz_1,$$

wobei drei mit den ursprünglichen Werten von a, b, c und z_2 berechnete M-Werte für drei Säulen eingesetzt wurden, bei den drei zu lösenden Gleichungen aber das gewünschte, zuerst ermittelte z_1 der jeweiligen Säule verwendet wurde.

In Tabelle IV sind für die untersuchten Stoffe auf den verwendeten Trennsäulen die Polaritätsfaktoren angegeben, sowie die mittleren Differenzen F_m zwischen berechneten und gemessenen M-Werten für die einzelnen stationären Flüssigkeiten

und Substanzen. Die mittlere Differenz zwischen gemessenem und berechnetem Wert beträgt für sämtliche Retentionszeiten 6.0 Einheiten.

VORAUSBERECHNUNG VON RETENTIONSZEITEN

Retentionszeiten bei 100° lassen sich nach der in dieser Arbeit verwendeten Formel:

$$I = I_0 + ax + by + cz$$

vorausberechnen. Wenn die drei Säulenpolaritäten (x,y,z) bekannt sind, müssen zur Voraussage einer Retentionszeit der Retentionsindex auf Squalan (I_0) und die drei Stoffpolaritäten (a,b,c) vorhanden sein. Für die Substanzen in Tabelle IV, die nicht auf allen Säulen gemessen wurden, sind diese Grössen bekannt. Es ist also möglich, 336 Retentionsindices dieser Stoffe auf 14 Säulen vorauszusagen. Zur Prüfung, wieweit solche Voraussagen erfüllt wurden, haben wir 20 Retentionsindices aus den berechenbaren durch Los ausgewählt und in einer dritten Versuchsserie gemessen. Die Ergebnisse von Messung und Berechnung sind in der Tabelle V zusammengestellt.

TABELLE V

VORAUSBERECHNUNG VON RETENTIONSZEITEN I

Säule	*Stoff*	$I_{berechnet}$	$I_{gefunden}$	*Differenz*
Carbowax 20 M	Methanol	904	893	11
	sec.-Butanol	1035	1008	28
Silikonnitril	Äthylbenzol	1127	1129	2
	Methylcyclohexan	782	800	18
Diäthylsuccinat	Butyraldehyd	1092	1099	7
	Methylenchlorid	1085	1076	9
Silikon 710	Äthylbenzol	959	953	6
	Pentanol-1	855	859	4
Marlophen 814	Toluol	969	973	4
	Methylcyclohexan	778	782	4
Fluorosilikon	Toluol	885	868	17
	Chlormethyl	439	440	1
Marlophen 87	Aceton	688	694	6
	3-Methylbutanol-1	1040	1033	7
Reoplex 400	Propanol	1079	1072	7
	Pentanol-3	1156	1149	7
Acetyl-tributylcitrat	Cumol	1055	1038	17
	Methylenchlorid	681	691	10
Carbowax Dioleat	Pentanol-1	1190	1179	11
	3-Methylbutanol-2	1035	1025	10
	mittlere Differenz F_m			11.3

Zur Bestimmung der Stoffpolaritäten ist es notwendig, die Substanz auf mindestens vier verschiedenen stationären Flüssigkeiten zu untersuchen. Für Dioxan und Phenylacetylen wurden die Werte für a, b und c aus den Retentionszeiten auf den Säulen Squalan, •Fluorosilikon, Polyäthylenglykol 1000 und Äthylenglykol-biscyanäthyläther berechnet und die hiermit errechneten Retentionsindices mit den gemessenen auf den anderen Säulen verglichen. Die Ergebnisse sind in Tabelle VI zusammengestellt.

Die Voraussage gelingt für die beiden Stoffe mit einem mittleren Fehler von 13 Einheiten nach KOVÁTS.

TABELLE VI

VORAUSBERECHNUNG VON M FÜR DIOXAN ($I_0 = 651$, $a = 23.95$, $b = 32.17$, $c = -0.26$) UND PHENYLACETYLEN ($I_y = 832$, $a = 13.48$, $b = 53.28$, $c = -0.73$)

	Säule	*Dioxan*			*Phenylacetylen*		
		berechnet	*gefunden*	*Diff.*	*berechnet*	*gefunden*	*Diff.*
12	Methyl-Phenyl-Silikonöl	123	123	0	114	120	6
13	Dioktylsebacat	112	107	5	146	133	13
14	Silikonöl DC 710	171	173	2	157	163	6
15	Fluorosilikon	234	234	—	161	161	—
16	Polypropylenglykol	200	205	5	266	240	26
17	Acetyltributylcitrat	213	211	2	243	238	5
18	Trikresylphosphat	268	252	16	299	283	16
19	Marlophen 87	265	273	8	329	306	23
20	Polypropylensebacat	282	285	3	325	318	7
21	Marlophen 814	318	320	2	397	380	17
22	Tween 80	344	352	8	424	405	19
23	Carbowax Dioleat	368	360	8	447	448	1
24	Silikon-Nitril	434	438	4	448	424	24
25	Carbowax 20 M	449	434	15	529	541	12
26	Carbowax 4000	457	443	14	539	550	11
27	PEG 1000	476	476	—	568	568	—
28	Reoplex 400	504	506	2	567	570	3
29	Diäthylengl. succ.	682	708	26	739	727	12
30	Äthylenglykolcyan.-	760	760	—	768	768	—
31	Tris.-Cyan.-	834	857	23	849	828	21
	Mittel F_m:			11.3			15.1

DISKUSSION

Die angegebene Berechnungsmethode für Retentionszeiten ergibt keine Aussagen über die Art der zwischenmolekularen Bindungen der untersuchten Zweistoffgemische. Die errechneten Stoff- und Säulenpolaritäten haben keinen direkten physikalischen Sinn, da diese Zahlen von den willkürlich angenommenen Polaritätsfaktoren der ersten beiden Stoffe auf den beiden Säulen am Beginn der Rechnung abhängig sind.

Dennoch sind folgende Zusammenhänge aus den Polaritätsfaktoren zu entnehmen:

(1) Substanzen, die ein Wasserstoffatom zur Wasserstoffbrückenbindung abgeben können, Alkohole, Chloroform, Methylenchlorid und Phenylacetylen, haben einen hohen Wert von b.

(2) Stoffe mit einer Karbonylgruppe (Aldehyde, Ketone und Ester) haben einen positiven Wert für c.

(3) Die Säule Fluorosilikon, die alle Karbonylverbindungen selektiv zurückhält, hat einen extrem hohen Wert für z.

(4) Die höheren Glieder homologer Reihen haben sehr ähnliche Polaritätsfaktoren. (Nach KOVÁTS[6] ist $I_p - I_0 = M$ für die höheren Glieder homologer Reihen konstant).

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ZUSAMMENFASSUNG

Über 600 bei einer Kolonnentemperatur von 100° gemessene Retentionszeiten lassen sich mit einem mittleren Fehler von 6 Einheiten nach KOVÁTS durch die Formel $I = I_0 + ax + by + cz$ darstellen. Zur Vorausberechnung von Retentionszeiten müssen von der stationären Flüssigkeit die Polaritätsfaktoren (x, y, z), vom Gelösten der Retentionsindex auf Squalan (I_0) und die Polaritätsfaktoren (a, b, c) bekannt sein. Die Polaritätsfaktoren wurden berechnet für 20 verschiedene stationäre Flüssigkeiten und für 42 Stoffe, darunter primäre, sekundäre und tertiäre, verzweigte, cyclische und ungesättigte Alkohole, Äther, Dioxan, Ester, Ketone, Aldehyde, Chlorkohlenwasserstoffe, Aromaten, cyclische, verzweigte und ungesättigte Kohlenwasserstoffe und Phenylacetylen. 54 Retentionszeiten von 18 Stoffen auf 17 stationären Flüssigkeiten wurden vorausberechnet und gemessen. Die mittlere Differenz betrug dabei 12.6 Einheiten nach KOVÁTS.

SUMMARY

More than 600 retention times, measured at a column temperature of 100°, can be expressed by the formula $I = I_0 + ax + by + cz$ with a mean error of 6 units, according to KOVÁTS. For the calculation, it is necessary to know the polarity factors of the stationary phases (x, y, z), and the retention index on squalane I_0 and the polarity factors a, b and c of the solutes. The polarity factors were determined for 20 different stationary phases and for 42 substances, comprising primary, secondary and tertiary, branched-chain, cyclic and unsaturated alcohols, an ether, dioxan, an ester, ketones, aldehydes, chlorinated hydrocarbons, aromatic, cyclic, branched-chain and unsaturated hydrocarbons and phenylacetylene. Fifty-four retention times of 18 substances on 17 stationary phases were calculated and measured experimentally. The mean difference amounted to 12.6 units according to KOVÁTS.

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GAS-CHROMATOGRAPHIC STUDY OF THE DECOMPOSITION OF THIO-CARBAMIC COMPOUNDS AS A FUNCTION OF TEMPERATURE

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INTRODUCTION

It is well known that the decomposition of thio-carbamic compounds in the cold and in acidic media can yield various sulphur compounds. In the specific case of ethylene bis-dithiocarbamates, the products may include hydrogen sulphide besides carbon disulphide and ethylenediamine sulphate, particularly if the decomposition is carried out below 100°.

The gas-chromatographic determination of carbon disulphide was studied in earlier investigations, which have already been reported by one of the present authors[1,2]. It was also desired to determine hydrogen sulphide and, possibly, to investigate other sulphur compounds which, according to the literature, might be formed in the decomposition of the thio-organic compounds in question. A method was therefore developed for the determination of hydrogen sulphide which would eliminate any interference by sulphur compounds other than hydrogen sulphide.

Many analytical methods have been described for the determination of hydrogen sulphide, carbon disulphide, mercaptans, alkyl sulphides, etc., in mixtures. Such methods are, however, rather difficult to carry out, and are not always completely reproducible.

Gas-chromatographic methods, which are based on physical rather than chemical characteristics, are particularly suitable, owing to their high sensitivity and to their selectivity, which permits the elimination of interference frequently encountered in other methods.

Excellent results were obtained using gas-chromatographic methods, by COOPE AND BRYCE[3] in their study of the pyrolysis of dimethyl disulphide, and by RYCE AND BRYCE[4] and EDWARDS *et al.*[5] in petrochemical analysis. The work on petrochemical analysis[4] was carried out with a view to obtaining a gas-chromatographic adsorption system specific for sulphur-bearing compounds. Of the various adsorbents tested, RYCE AND BRYCE[4] found that tricresyl phosphate on Celite, in the proportions recommended by JAMES AND MARTIN[6], was suitable for the fractionation of acidic sulphur compounds.

In the above-mentioned gas-chromatographic investigation on carbon disulphide[1,2], it was observed that, below the temperature recommended by CLARKE[7] (100°), the yield of carbon disulphide varied according to the temperature at which the dithiocarbamates were distilled.

There is no general agreement regarding the products which may be formed during the decomposition of these compounds.

In the present work it was intended to examine, by a gas-chromatographic method, such decomposition as a function of temperature. For such an investigation a reliable method was necessary and if possible simultaneous determination of hydrogen sulphide and carbon disulphide would be advantageous.

DETERMINATION OF HYDROGEN SULPHIDE AND CARBON DISULPHIDE

Preliminary experiments showed that tricresyl phosphate perfectly fulfilled the requirements for the case in question as the stationary phase, and was also found to be suitable not only for hydrogen sulphide, as shown by RYCE AND BRYCE[4], but also for carbon disulphide.

In contrast to most literature reports concerned with the investigation of gaseous mixtures, the object of the present work was the gas-chromatographic analysis of substances displaced as gases from solids or solutions by chemical reactions. Such gases should be condensed at low temperatures, to allow their rapid introduction *in toto* into the adsorption equipment for the quantitative determination.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF HYDROGEN SULPHIDE BY GAS CHROMATOGRAPHY

Apparatus	Fractovap model B (C. ERBA-Milan)
Support	Celite C22, 30–60 mesh
Column	Length 4 m; I.D. 0.6 cm
Stationary phase	Tricresyl phosphate (25 %)
Carrier gas	Helium
Current	5.3 l/h
Temperature of thermostat chamber	20° ± 0.2°
Bridge current	8 mA
Speed of recording chart	1.25 cm/min
Retention time of hydrogen sulphide	5 min 12 sec

The optimum retention time, which has permitted the complete isolation of hydrogen sulphide, and hence its reliable detection, as well as the measurement of the corresponding peak without the need for extrapolation, can be obtained by working at 20° under a helium current of 5.3 l/h. Table I shows the experimental conditions used, and an example of the hydrogen sulphide peaks obtained is reproduced in Fig. 1.

Hydrogen sulphide used for the calibration was produced from alkaline solutions (5 % sodium hydroxide) containing known concentrations of sodium sulphide, from which samples were removed with a microsyringe to introduce the desired quantity of the material into the distillation apparatus.

The hydrogen sulphide was evolved by means of sulphuric acid, and was condensed from a current of helium carrier gas, in a liquid air trap, where it solidified together with other products which possibly included small quantities of water vapour. The trap was then connected to the gas chromatography equipment (ERBA Fractovap model B), and the contents were vaporised and transferred to the adsorption column by entrainment in a current of helium.

To meet the requirements of this work the design of the trap must be such that it is sufficiently accurate in operation to permit the determination of hydrogen sulphide in the concentration range of 10^{-7} to 10^{-4} moles per litre.

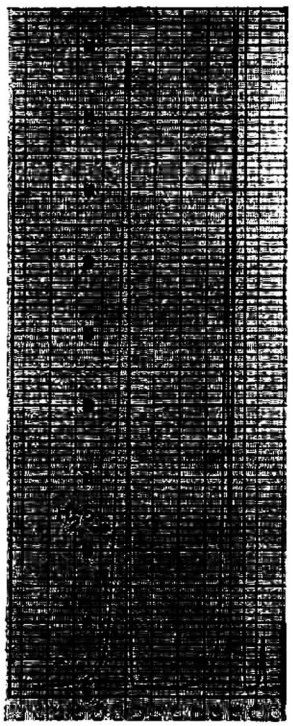


Fig. 1. Hydrogen sulphide peak.

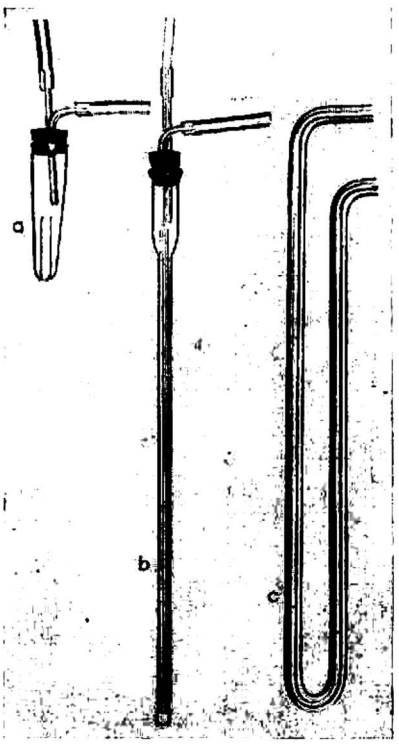


Fig. 2. Types of trap used.

Investigation of types of trap

Fig. 2 shows three types of traps studied for suitability in the present work. Type (a) was inserted directly into the chromatographic apparatus in an attempt to simplify the displacement of the hydrogen sulphide, by avoiding the need for distillation and trapping in liquid air, which takes about an hour. This trap is so designed that direct contact between the reagents is achieved at the desired moment. However, owing to the excessive quantity of water vapour displaced during the reaction and the slowness of the latter, the use of this trap is not recommended, particularly in the case of quantitative work.

Trap (c), which has a capillary section and has been described in an earlier note[8], was also found to be unsuitable for this work; when it was inserted directly into the end of the distillation apparatus, as illustrated earlier[8], or totally immersed in liquid air or nitrogen in a Dewar flask, it allowed appreciable quantities of the gas being examined to escape before it had completely solidified. It should be remembered that the distillation was carried out with gentle suction in a current of helium.

The peculiar structure of trap (b) permitted a gradual cooling of the hydrogen sulphide, and solidification along the internal capillary tube coaxial with the outer envelope. A cadmium acetate detector, buffered to pH 6 and inserted immediately after the trap, gave no indication of any escape of gas, in contrast to the behaviour observed with the U-shaped trap (c).

Using sodium sulphide solutions, made alkaline as described above to avoid hydrolysis of the sulphide, it was possible to construct a calibration curve, shown in Fig. 3, from the results listed in Table II.

It can be seen that the range of concentration which can be quantitatively analysed by planimetric measurement of areas under the peaks lies between 10 and 1000 γ. It was considered unnecessary to extend the curve to higher concentrations, since other less sensitive methods could be used in such cases.

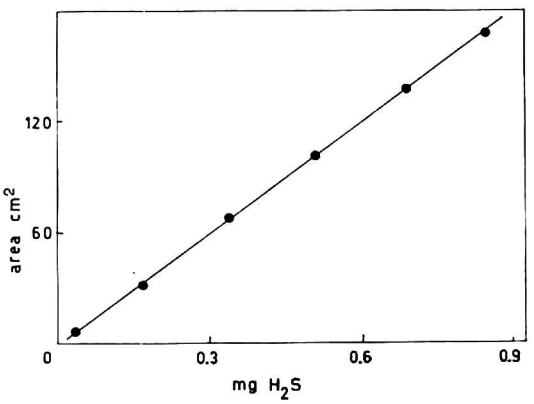


Fig. 3. Calibration curve for hydrogen sulphide.

Although the gas-chromatographic technique adopted in the present work reduced the limits of detection for hydrogen sulphide to about 3–4 γ, such results are, however, of only qualitative value, since appreciable errors are introduced in the determination of the areas under the peaks at these concentration levels.

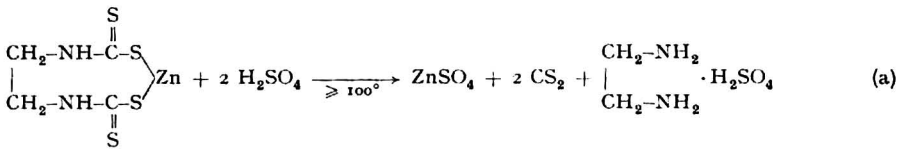
TABLE II

DATA RELATING HYDROGEN SULPHIDE CONCENTRATION WITH PEAK AREA

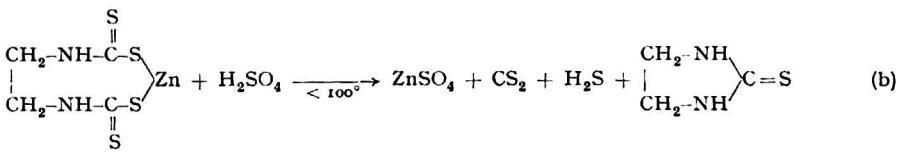
mg of hydrogen sulphide	*Area under the peak in cm²*
0.034	6.8
0.170	33.2
0.340	68.0
0.510	102.8
0.680	136.4
0.850	168.5

INVESTIGATION OF DECOMPOSITION REACTION

As is known from the literature[7], the decomposition of ethylene bis-dithiocarbamates can proceed by two different paths, according to the temperature:



(a)



(b)

Earlier work carried out by one of the present authors[1,2,8] was concerned with the development of a gas-chromatographic method, based on reaction mechanism (a), for the determination of CS_2 in such a way as to eliminate any source of error resulting from the possible presence in the system of other interfering sulphur compounds. Mechanism (b) was considered in the present work, the aim being to determine, under otherwise identical conditions, the temperature at which reaction (b) leading to the formation of hydrogen sulphide could be detected to the greatest extent.

The first step was naturally to prepare zinc ethylene bis-dithiocarbamate in the purest possible state, particularly as regards sulphur to ensure that the hydrogen sulphide evolved during the acid decomposition was not even partly derived from sulphur present in any form as an impurity in the finished product.

The method used in the preparation of the compounds in question has been reported in an earlier paper[9] relating to investigations carried out by one of the authors on the amino-base constituents of dithiocarbamic acid derivatives.

With regard to the ethylene thiourea reported among the decomposition products of reaction (b) above, it was shown chromatographically that this compound could not be found in the residue from the distillation of acidic solutions of the ethylene bis-dithiocarbamates under an atmosphere of helium.

When pure ethylene thiourea is subjected to the same treatment with thiourea at the same temperature as that at which the various distillation residues are obtained, it gives an R_F value, on chromatographic examination, exactly the same as that obtained by chromatography of pure ethylene thiourea.

Ethylene thiourea can also be detected when pure ethylene bis-dithiocarbamates, not subjected to any previous treatment, are chromatographed under the conditions reported below (see Table III). It thus appears that the ethylene thiourea is already present in the ethylene bis-dithiocarbamates, and is not formed during the thermal decomposition of the dithio compound with sulphuric acid, so that this decomposition must give rise to sulphur compounds of another type. The experimental conditions shown in Table III were derived, with some modifications, from those described for an earlier investigation carried out at this Institute[10].

TABLE III

EXPERIMENTAL CONDITIONS AND R_F VALUES FOR CHROMATOGRAPHY OF ETHYLENE THIOUREA AND THIOUREA

Method	Descending, on paper
Paper	Whatman 3 MM
Developer	Isobutyl alcohol–isopropyl alcohol–formic acid–water (40:30:10:20)
Temperature	22°
Time	12 h
Detector	Ammoniacal solution of silver nitrate
R_F for ethylene thiourea	0.72
R_F for thiourea	0.55

In order to avoid even slight losses in the determination of the hydrogen sulphide, we thought of substituting the switching apparatus supplied for the chromatographic apparatus for the introduction of the gaseous products with another device to eliminate the disadvantage of inevitable dead spaces. The preferred method was to insert the condensation cell in the sockets of the apparatus by a fairly simple device consisting of two suitably clamped glass Y-pieces fitted with polythene connections (Fig. 4).

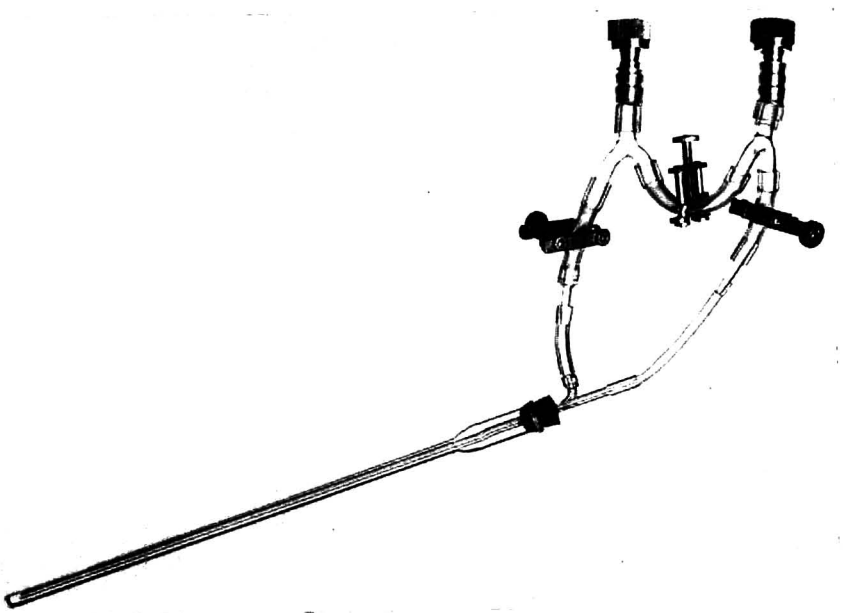


Fig. 4. Trap with connection to chromatographic apparatus.

Effect of temperature

The products of acidic decomposition of the ethylene bis-dithiocarbamates were distilled for about 1 h in a current of pure nitrogen to avoid any possible oxidation, at 50°, 60°, 70°, 80° and 90°. The sulphuric acid used in all tests was 1.1 *N*, *i.e.* at

the concentration recommended for CLARKE's method[7] for the determination of dithiocarbamic acid derivatives.

To facilitate following the course of the decomposition of the thio-organic compounds in question, the hydrogen sulphide and carbon disulphide were detected simultaneously by gas chromatography, under the conditions described earlier for hydrogen sulphide. For this purpose, it was sufficient to construct a calibration curve for carbon disulphide at 20°, which is shown in Fig. 5 and which was derived

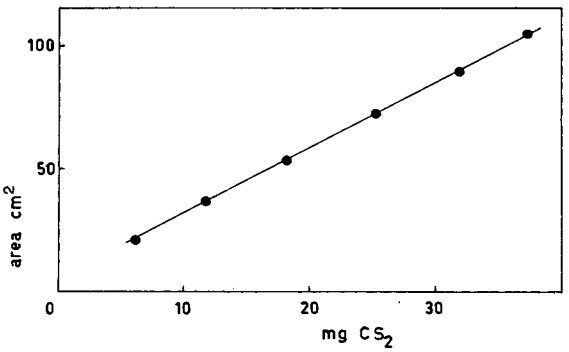


Fig. 5. Calibration curve for carbon disulphide.

from the data given in Table IV. The concentrations of carbon disulphide, which had solidified from time to time together with the hydrogen sulphide in the trap immersed in liquid air during distillation, were obtained from this curve. The retention time of the carbon disulphide increased under the conditions described to 45 min, as compared with 9 min 45 sec at 80° in the investigation mentioned earlier[1].

TABLE IV

DATA FOR CONSTRUCTION OF CALIBRATION CURVE FOR CARBON DISULPHIDE

mg of carbon disulphide	*Area under peak in cm²*
6.0	11.0
12.7	36.2
19.1	53.4
25.5	72.5
31.8	89.0
37.2	105.3

Simultaneous detection of the two sulphur compounds speeded the determination of their respective concentrations and of their ratio. It was then possible to verify whether, and under what conditions, the molar ratio between hydrogen sulphide, carbon disulphide, and ethylene thiourea was effectively 1:1:1, as reported in the literature, *i.e.* according to reaction (b) above.

Reaction products and their molar ratios

Table V shows the percentages of the various sulphur compounds, deduced

TABLE V

PERCENTAGE OF CS_2, H_2S AND OTHER MONOSULPHUR COMPOUNDS AS A RESULT OF DECOMPOSITION OF ETHYLENE BIS-DITHIOCARBAMATES AT VARIOUS TEMPERATURES

Decomposition temperature	*Per 25.694 mg of sulphur*	%
50°	17.519 mg as carbon disulphide	68.18
	0.020 mg as hydrogen sulphide	0.08
	8.155 mg as other monosulphur compounds	31.74
60°	20.214 mg as carbon disulphide	78.67
	0.016 mg as hydrogen sulphide	0.08
	5.464 mg as other monosulphur compounds	21.26
70°	20.908 mg as carbon disulphide	81.37
	0.021 mg as hydrogen sulphide	0.08
	4.765 mg as other monosulphur compounds	18.55
80°	21.477 mg as carbon disulphide	83.59
	0.038 mg as hydrogen sulphide	0.15
	4.179 mg as other monosulphur compounds	16.26
90°	24.509 mg as carbon disulphide	95.39
	0.038 mg as hydrogen sulphide	0.15
	1.147 mg as other monosulphur compounds	4.46

TABLE VI

VARIATION OF MOLAR RATIOS OF REACTION PRODUCTS WITH TEMPERATURE

Decomposition temperature	*Moles*	*Molar ratio*
50°		
Ethylene thiourea	0.255	1
Carbon disulphide	0.274	1.07
Hydrogen sulphide	0.0005	0.002
60°		
Ethylene thiourea	0.171	1
Carbon disulphide	0.316	1.85
Hydrogen sulphide	0.0005	0.003
70°		
Ethylene thiourea	0.149	1
Carbon disulphide	0.327	2.19
Hydrogen sulphide	0.0005	0.003
80°		
Ethylene thiourea	0.131	1
Carbon disulphide	0.336	2.56
Hydrogen sulphide	0.001	0.008
90°		
Ethylene thiourea	0.036	1
Carbon disulphide	0.386	10.64
Hydrogen sulphide	0.001	0.028

from the sulphur concentrations due to each of the compounds determined by gas chromatography, and related to the total sulphur content of the dithiocarbamate analysed from time to time.

From the results, it was possible to deduce that at 50° the molar ratio of reaction (b) approached the value of 1:1 for the carbon disulphide and ethylene thiourea (or a compound containing one atom of sulphur) whilst the molar ratio of hydrogen sulphide with respect to the other two compounds was found to be 0.002. At 80° the molar ratios between ethylene thiourea, carbon disulphide, and hydrogen sulphide become 1:2.5:0.007, changing at 90° to 1:10.6:0.03. The molar ratios at the various temperatures are shown in Table VI.

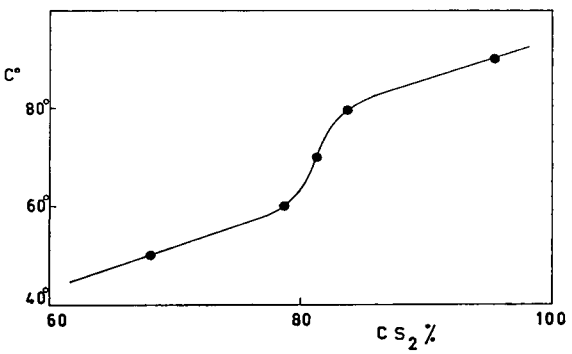


Fig. 6. Percentages of carbon disulphide at various temperatures.

Even assuming that the fraction of sulphur other than that due to carbon disulphide and hydrogen sulphide is a constituent of the ethylene thiourea, or at least of some compound containing one atom of sulphur (mercaptan or isothiocyanate), the reaction in question only gives a ratio of 1:1 around 50°, and then only for carbon disulphide and the monosulphur compound, whatever the latter may be. The molar ratio of hydrogen sulphide corresponding to reaction (b) was never observed at temperatures between 50° and 90°.

Fig. 6 shows a graph of the percentages of carbon disulphide found by analysis, as a function of the decomposition temperature of the dithiocarbamate. It can be seen that the variation follows the same course below 60 and above 80°, being rather less pronounced between these temperatures.

SUMMARY

A description is given of the experimental procedure developed for the simultaneous determination of hydrogen sulphide and carbon disulphide, evolved during the decomposition of metal ethylene bis-dithiocarbamates in acid media, at temperatures between 50° and 100°. The limits of sensitivity obtained with the apparatus used for the quantitative determination of these gaseous products are described.

The various reaction mechanisms proposed for the decomposition of the ethylene bis-dithiocarbamates are considered, and the molar ratios of the experimentally obtained sulphur compounds in relation to the total sulphur content of the original thio-organic compound are discussed.

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THE SIMULTANEOUS DETERMINATION OF C, Cl, Br AND I IN ORGANIC COMPOUNDS BY GAS CHROMATOGRAPHY

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INTRODUCTION

With the advent of gas chromatography, DUSWALT AND BRANDT[1] and SUNDBERG AND MARESH[2] developed methods of determining C and H. SCOTT *et al.*[3] modified the method to determine O_2 and N_2. SUNDBERG AND MARESH[2] using the same principles determined C, H and N as did NIGHTINGALE AND WALKER[4] using a high frequency induction furnace and $AgMnO_4$ as an oxidizing agent. BEUERMAN AND MELOAN[5,6] adapted a combination of the Pregl catalytic combustion method with gas chromatography for the simultaneous determination of C and S.

Inorganic halogen systems have been studied previously[7–12] but organic studies are not as plentiful. Because of the fast analysis times possible with a catalytic combustion, it was believed that C, S, N, Cl, Br and I could possibly be determined simultaneously if a chromatographic separation was added. The results of this study indicate that this may be possible in practice.

EXPERIMENTAL

Apparatus

The apparatus was the same as that used by BEUERMAN AND MELOAN[5].

Chemicals

Carbon dioxide. Carbon dioxide was prepared by the acidification of reagent grade sodium carbonate. The liberated gas was trapped in liquid nitrogen together with some air. The trapped gas was allowed to vaporize and then flushed into the column to determine its retention time.

Chlorine. Chlorine was prepared by acidification of sodium hypochlorite.

Bromine. Liquid bromine was used. The bromine vapors were trapped in liquid nitrogen. Gentle vacuum was used to help collect the vapors into the trap. The same procedure for the above gases was followed for the trapped vapors.

Combustion of halogen containing organic compounds. Organic compounds containing chlorine, bromine and iodine were combusted in platinum boats at a temperature of 800° and their combustion gases trapped in liquid nitrogen. The trapped gases were allowed to vaporize and then passed into the column. The retention times of the

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combustion gases were compared with the retention times of the prepared gas samples. Organic compounds containing chlorine and bromine, chlorine and sulfur, hydrogen, and oxygen, in the same compound were also combusted.

For the quantitative aspect of the work, the organic compounds were purified either by sublimation, crystallization from their saturated solutions, or distillation. The purified compounds were dried over phosphorus pentoxide. The purified and dried samples were kept in small tightly stoppered vials. The compounds were weighed to the nearest 0.001 of a milligram on an Ainsworth microbalance. The area of the peaks in the chromatograms obtained from the combustion gases of the compounds were measured with a Keuffel and Esser Co. No. F4236 planimeter.

RESULTS AND DISCUSSION

The results of the columns prepared are tabulated in Table I.

From the results shown in Table I, it was decided to use the column with 33 % silicone stopcock grease (Dow-Corning) on Chromosorb P, acid-washed (30–60), at 68° with a helium flow rate of 24 ml/min. The retention times of the combustion gases of some organic halogen compounds are shown in Table II.

TABLE I

RETENTION TIMES OF THE GAS SAMPLES

Column description: *Liquid phase* *Column support* *Tube material* *Length and O.D.*	*Chromatograph parameters*		*Retention time (min)*				
	Temp. (°C)	*Flow rate (ml/min)*	O_2	CO_2	SO_2	Cl_2	Br_2
33 % fluorolube grease (LG-160) Columpak (30–60)							
20 ft. Cu, O.D. $^1/_4$ in.	120	33	2.4	2.75	2.9	2.9	*
16 ft. Ni, O.D. $^1/_4$ in.	60	33	1.8	2.00	2.8	3.0	8.3
22.5 ft. Al, O.D. $^1/_4$ in.	50	33	3.0	3.4	4.4	5.3	15.5
33 % fluorolube grease (LG-160) Chromosorb W (80–100)							
30 ft. Al, O.D. $^1/_4$ in.	90	20	7.4	8.0	9.1	11.1	
30 % fluorolube grease (LG-160) Columpak (30–60)							
13 ft. Cu, C.D. $^1/_4$ in.	30	33	1.6	1.75	2.8	2.8	*
	70	33	1.6	1.75	2.2	2.2	*
33 % dinonyl phthalate Columpak (30–60)							
5 ft. stainless steel	50	33	0.6	0.6	2.0	1.8	
2.5 % Kel-F No. 40 on glass beads (120–170)							
6 ft. Cu, O.D. $^1/_4$ in.	100	33	At this flow rate and temperature, there was no separation of O_2 and CO_2				

(*continued on p. 25*)

TABLE I (*continued*)

Column description: *Liquid phase* *Column support* *Tube material* *Length and O.D.*	*Chromatograph parameters*		*Retention time (min)*				
	Temp. (°C)	*Flow rate (ml/min)*	O_2	CO_2	SO_2	Cl_2	Br_2
2.5 % silicone stopcock grease (Dow-Corning) Columpak (30–60) 12 ft. Al, O.D. $^1/_4$ in.	50	33	No separation of O_2 and CO_2				
15 % silicone stopcock grease (Dow-Corning) Columpak (30–60) 12 ft. Al, O.D. $^1/_4$ in.	30	33	1.6	1.8	2.45	3.30	21.5
	70	33	1.4	1.4	1.7	2.1	6.2
15 % silicone stopcock grease(Dow-Corning) Columpak (30–60) 12 ft. Al, O.D. $^1/_4$ in.	120	33					3.4
25 % silicone stopcock grease (Dow-Corning) Columpak (30–60) 18.5 ft. Cu, O.D. $^1/_4$ in.	50	33	2.1	2.4	3.4	3.8	*
	120	33	1.9	2.2	2.6	2.8	*
12 ft. Al, O.D. $^1/_4$ in.	45	33	1.1	1.3	1.7	2.4	13.2
	60	33	1.1	1.1	1.6	1.9	8.9
	140	28	1.6	1.6	1.6	1.6	3.7
	150	25					3.5
30 ft. Al, O.D. $^1/_4$ in.	85	33	3.0	3.3	4.8	5.5	
	100	33	3.0	3.2	3.7	4.6	
33 % silicone stopcock grease (Dow-Corning) Chromosorb W (80–100) 12 ft. 2 in. Ni, O.D. $^1/_4$ in.	50	33	2.1	2.4	3.1	5.7	12.8
33 % silicone stopcock grease (Dow-Corning) Chromosorb P, acid-washed (30–60) 30 ft. Al, O.D. $^1/_4$ in.	65	25	4.2	4.9	7.0	10.5	
	68	24	4.3	5.0	7.5	11.0	40.0
	68	23	4.5	5.5	7.7	12.0	44.0
	70	25	4.0	4.5	6.2	8.0	
	80	25	3.8	4.3	5.5	7.8	

* No peak after 1 h.

The retention times of the combustion gases of the halogen containing compounds coincided with those for pure chlorine gas and bromine gas.

The combustion of iodine containing organic compounds showed iodine crystals depositing in the trap. Due to the difficulties encountered in determining the iodine vapors by means of the type of gas chromatograph used and the techniques involved, work on the determination of iodine vapors was discontinued. A type of programmed temperature gas chromatograph would be suitable for the simultaneous determination of chlorine, bromine and iodine vapors.

TABLE II

RETENTION TIMES OF THE COMBUSTION GASES

Compound	*Chromatograph parameters*		*Retention time (min)*					
	Temp. (°C)	*Helium flow rate (ml/min)*	O_2	CO_2	SO_2	N_2	Cl_2	Br_2
Monochloroacetic acid	65	25	4.2	4.9			10.5	
2,4-Dichlorobenzoic acid	68	24	4.2	4.9			11.0	
2,4-Dichloroquinoline	68	24	4.3	5.0		10.0	10.8	
p-Toluenesulfonyl chloride	68	24	4.2	5.0			11.0	
1-Bromo-3-chloropropane	68	24	4.3	5.0			11.0	40.0
p-Bromochlorobenzene	68	24	4.3	5.0			11.0	40.0
4-Chlorophenyl sulfone	68	25	4.0	4.5	7.3		9.7	
Trichloroacetic acid	70	25	4.0	4.5			8.0	
Trichloroacetic acid	80	25	4.0	4.4			7.8	

No evidence of chlorine oxides or bromine oxides was shown in the chromatograms. If ever the respective oxides were formed, they must have decomposed to the respective halogens and oxygen at the temperature of combustion of 800°. In general, the halogen oxides are known for their instability[13].

Chlorine monoxide, Cl_2O, explodes on heating to a mixture of chlorine and oxygen. Chlorine dioxide, ClO_2, is extremely reactive. In the pure state it explodes violently and therefore has to be diluted with carbon dioxide or air for safe handling. Chlorine hexoxide, Cl_2O_6, decomposes appreciably to chlorine dioxide and oxygen even at the melting point. Chlorine heptoxide, Cl_2O_7, detonates when heated or subjected to shock. The existence of chlorine tetroxide, ClO_4, is only postulated by GOMBERG[13] but not yet proven.

The oxides of bromine have properties similar to the oxides of chlorine. Bromine monoxide, Br_2O, decomposes into bromine and oxygen at any temperature above —40°. Bromine dioxide, BrO_2, decomposes to bromine and oxygen at higher temperatures (above —40°).

The formation of hydrogen chloride and hydrogen bromide occurred when the oxygen flow rate in the combustion tube was slow, but upon increasing the oxygen flow rate, the hydrogen chloride reacts to form water and chlorine, and the hydrogen bromide to water and bromine. Hydrogen chloride is converted to chlorine and water when heated in an atmosphere of oxygen in the presence of catalysts even at temperatures of 450°[13]. The hydrogen chloride and the hydrogen bromide peaks come after the carbon dioxide peak.

Considering the dissociation constant of chlorine gas which is 10^{-8} and that of bromine gas, 10^{-3} at 1000°, it is then seen that these gases could exist as such at temperatures of 750–800°.

There was no evidence of a BrCl peak in the chromatograms of the combustion gases of organic compounds containing chlorine and bromine. The interhalogen, BrCl, that may have formed, may have been dissociated into bromine and chlorine at the temperature of combustion. Furthermore, bromine monochloride undergoes photochemical decomposition. Its instability is indicated by its small heat of formation which is $+0.75 \pm 0.5$ kcal/mole[13].

The formation of sulfur dioxide from the combustion of 4-chlorophenyl sulfone was evident but for the combustion products of benzenesulfonyl chloride and *p*-toluenesulfonyl chloride, the sulfur dioxide peak was missing. This may be due to a lower combustion temperature used (800° for the short furnace and 750° for the long furnace). Most of the sulfur dioxide that may have formed may have been converted to sulfur trioxide. No attempts were made to verify the sulfur trioxide peak.

It was found that the oxygen flow rate of the combustion tube is critical not only because of the formation of hydrogen chloride and hydrogen bromide but also of the possibility of the newly formed halogens attacking the platinum catalysts if they are not removed right away. It was observed that in the combustion of compounds with a large number of chlorine atoms, like hexachloroethane and hexachlorobenzene, there was a reddish-brown deposit at the end of the combustion tube. This deposit must have come from the reaction of chlorine and the platinum catalysts to form platinum tetrachloride, which is a reddish-brown compound. Increasing the oxygen flow rate by applying a slight vacuum and lowering the temperature of the long furnace to 750–800°, gave no such deposit.

Based on the results of the performance of the different columns, it was noted that copper and stainless steel cannot be used for bromine determinations because these metals hold bromine vapors tenaciously especially in the presence of even a trace of moisture. Nickel and aluminum were found to be able to resolve a mixture of bromine and air. Shorter columns of nickel and aluminum can be utilized to determine chlorine and bromine if the separation of carbon dioxide from oxygen is not to be considered.

The utmost precautions were taken to prevent water from getting inside the trap and the gas chromatograph because the presence of water leads to erratic results due to its reaction with the combustion gases.

The oxygen flow rate was adjusted from 15–20 ml/min in the combustion train to insure complete combustion of the sample, and at the same time oxidize all the organic halogens to their molecular forms. Nickel, monel tubing and teflon were found to work with chlorine and bromine. It was found in this work that aluminum could be used too, provided the prepared column is conditioned for several days at some elevated temperatures (about 70–100°) and the determinations are made under completely anhydrous conditions. This may be due to the formation of a film of aluminum oxide on the aluminum surface. The film of aluminum oxide is inert to the dry halogen gases.

Evaluation of the method

The results of the analysis of a number of organic compounds containing chlorine and bromine are shown in Tables III and Table IV.

A linear relationship was obtained between area of peak and milligrams of chlorine and bromine in the sample from the analysis of different sample weights.

The average deviation calculated for the chlorine determinations (n = number of determinations) between the theoretical and the determined percentages of chlorine is:

$$\text{Average deviation} = \frac{\text{deviations}}{n} = \frac{5.19}{18} = 0.29\,\%.$$

TABLE III

EVALUATION OF THE METHOD FOR DETERMINING ORGANIC CHLORINE

Compound	*Weight of sample (mg)*	*Area of peak (sq. in.)*	*Cl present (mg)*		*Cl (%)*	
			Theory	*Found*	*Theory*	*Found*
Dichloromethane	4.755	5.12	3.970	3.968	83.49	83.45
	4.995	5.38	4.170	4.170	83.49	83.49
Trichloroacetic acid	3.720	3.12	2.422	2.418	65.10	65.00
	3.041	2.55	1.980	1.976	65.10	64.99
Monochloroacetic acid	4.310	2.06	1.617	1.597	37.52	37.05
	4.510	2.14	1.692	1.658	37.52	36.63
Hexachlorobenzene	5.122	4.93	3.826	3.821	74.70	74.60
	4.875	4.69	3.642	3.677	74.70	74.40
Chlorobenzene	4.100	1.64	1.291	1.271	31.50	31.00
	3.560	1.44	1.121	1.116	31.50	31.35
2,4-Dichlorobenzoic acid	2.553	1.22	0.948	0.945	37.12	37.02
	3.410	1.59	1.266	1.232	37.12	37.13
Benzenesulfonyl chloride*	5.080	1.31	1.019	1.015	20.06	19.98
	5.344	1.38	1.072	1.068	20.06	19.98
4,7-Dichloroquinoline**						
1-Bromo-3-chloropropane	6.653	1.91	1.498	1.480	22.52	22.25
	5.512	1.58	1.241	1.225	22.52	22.22
p-Bromochlorobenzene	5.456	1.27	1.010	0.984	18.52	18.04
	3.440	0.81	0.637	0.628	18.52	18.26

* No sulfur dioxide peak in the chromatogram; sulfur dioxide may have been converted to sulfur trioxide.

** Chlorine peak not fully resolved from an unknown peak which may be an oxide of nitrogen, either nitrogen dioxide or nitric oxide.

The average deviation calculated for the bromine determinations is:

$$\frac{4.02}{16} = 0.25\,\%.$$

The method just discussed may be used to determine carbon, chlorine and bromine simultaneously and if programmed temperature is used, then iodine as well.

No attempts were made to identify the nitrogen peak to see what oxide if any was present nor was any attempt made to look further into why an SO_2 peak occurred sometimes and not others. The study of these latter two problems is now underway and since the S and N peaks can be separated from the rest (see Table II) it now seems possible to be able to do C, S, N, Cl, Br and I simultaneously in the not too distant future. If the water that was previously absorbed could be regenerated the hydrogen could also be determined.

It should also be mentioned that if a high frequency induction furnace is used then the need for a trap is eliminated, and the analysis time can be reduced 5–10 min.

TABLE IV

EVALUATION OF THE METHOD FOR DETERMINING ORGANIC BROMINE

Compound	*Weight of sample (mg)*	*Area of peak (sq. in)*	*Br present (mg)*		*Br (%)*	
			Theory	*Found*	*Theory*	*Found*
m-Bromotoluene	4.819	0.92	2.251	2.249	46.71	46.67
	4.190	0.82	1.957	1.956	46.71	46.68
Bromoethane	5.638	1.69	4.134	4.132	73.33	73.29
	5.470	1.64	4.011	4.009	73.33	73.29
1-Bromoheptane	3.581	0.65	1.598	1.589	44.62	44.37
	3.585	0.65	1.600	1.589	44.62	44.32
o-Bromobenzoic acid	7.343	1.19	2.919	2.910	39.75	39.63
	7.440	0.55	1.367	1.345	39.75	39.10
1-Bromonaphthalene	3.318	0.52	1.280	1.271	38.59	38.31
	5.862	0.92	2.262	2.249	38.59	38.36
1,1,1-Tribromo-2-methyl-propanol	6.471	2.02	4.991	4.976	77.13	76.90
	6.558	2.05	5.058	5.017	77.13	76.50
1-Bromo-3-chloropropane	6.653	1.37	3.377	3.350	50.76	50.35
	5.512	1.14	2.798	2.787	50.76	50.56
p-Bromochlorobenzene	5.456	0.93	2.280	2.274	41.78	41.68
	3.440	0.58	1.437	1.421	41.78	41.31

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SUMMARY

Organic halogen compounds were combusted at 800° to CO_2, H_2O, Cl_2, Br_2 and I_2 using a modified Pregl method. H_2O was removed using anhydrous $CaSO_4$, and the remaining gases were trapped at liquid N_2 temperatures. The trapped gases were vaporized and the components separated by gas chromatography. 33% silicone stopcock grease on Chromosorb P, acid-washed (30–60) was used at 68° with He as a carrier gas. O_2 and S do not interfere. The relative standard deviation is less than $\pm 0.3\%$.

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GAS CHROMATOGRAPHY OF VOLATILE AMINO ACID DERIVATIVES

I. ALANINE, GLYCINE, VALINE, LEUCINE, ISOLEUCINE, SERINE AND THREONINE

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INTRODUCTION

Gas chromatographic methods of analysis are increasingly being used for biological problems because of their speed and convenience, and their application to the analysis of the amino acids is receiving much attention. In particular it is of great potential value for peptide analysis.

The conversion of the amino acids to suitable volatile derivations has been achieved in a number of ways: conversion of the amino acids to the corresponding aldehyde by the action of ninhydrin[1–5], decarboxylation to the corresponding amines[6], conversion of the amino acid to the α-chloro derivative[7] and to the α-hydroxy derivative[8,9]. Amino acid methyl esters have also been used[10,11]. A number of N-trimethylsilyl amino acid trimethylsilyl esters were prepared and separated by RÜHLMANN AND GIESECKE[12]. N,N-Dimethyl amino acid methyl esters have been investigated[13]. YOUNGS used the N-acetyl amino acid *n*-butyl esters[14] while the N-acetyl *n*-amyl esters (and the N-acetyl esters of some of the lower alcohols) were studied extensively by JOHNSON, SCOTT AND MEISTER[15] and by SHLYAPNIKOV, KARPEISKY AND LITVIN[16]. GRAFF, WEIN AND WINITZ[17] prepared the N-acetyl *n*-propyl esters. LOSSE, LOSSE AND STÖCK[18] prepared and separated some N-formyl amino acid methyl esters. The N-trifluoroacetyl (TFA) amino acid methyl esters were prepared by a number of workers[19–25], whilst N-TFA *n*-butyl esters were used by ZOMZELY, MARCO AND EMERY[26]. Like ourselves[13,27], TEUWISSEN, LENAIN, DORLET AND LEONIS[28] obtained best results with N-TFA amino acid *n*-amyl esters. PISANO, VANDENHEUVEL AND HORNING[29] chromatographed a number of phenylthiohydantoin derivatives of amino acids, as well as their N-2,4-dinitrophenyl methyl esters (see also refs. 30, 31).

Since the ultimate objective is to develop a method which is capable not only of identifying but also of estimating the amino acids in a mixture, quantitative aspects have had prior consideration in our choice of derivatives. The final choice was based on considerations of (a) applicability to all the common α-amino acids, (b) volatility, (c) yield, (d) ease of separation and (e) practical convenience in making and handling the derivatives.

The present work reports the results of an investigation of the most volatile of the derivatives used by us (the TFA amino acid *n*-amyl esters) namely those of alanine, glycine, valine, leucine, isoleucine, serine and threonine.

Temperature programming was not used, because the nature of the detector, which we consider to be the most satisfactory for quantitative work, restricted us to isothermal separations. This has involved a search for a stationary phase which enables these seven derivatives to be separated unequivocally and without oves-lapping of peaks. All phases we have tried are mentioned, since some of the novel oner might prove useful in other applications, being suitable in all senses except the specific retention properties needed for this work, while others can usefully be excluded from further consideration.

MATERIALS AND METHODS

Abbreviations

DDS = dichlorodimethylsilane

H.E.T.P. = height equivalent to a theoretical plate calculated from the formula:

$$h = 330 \left(\frac{\text{width at half height}}{\text{relative retention time}} \right)^2 \text{ for the columns used.}$$

PVP = polyvinylpyrrolidone

TFA = trifluoroacetyl.

Apparatus

A D6 gas chromatograph (Griffin & George Ltd., Alperton, Middlesex, Great Britain) with a ga-density balance detector was used. Columns consisted of two stainless steel tubes of 5 mm I.D., connected at their lower end by a stainless steel capillary U-tube. Packed length was 182 cm. Nitrogen (99.9 % "White spot", British Oxygen Co., Wembley, Middlesex, Great Britain) was used as carrier gas. Integrations were carried out with an Instron Integrator (Instron Ltd., High Wycombe, Bucks. Great Britain).

Preparation of the packed columns

(*a*) *General.* It was found necessary to pretreat supports to prevent tailing of the peaks. Glass beads were tried, but the peaks obtained on such packings showed poor efficiency, even when fine Celite (Johns-Manville "Super-floss", L. Light & Co. Ltd., Colnbrook, Bucks.) was added[32]. Fluoropak 80 used as a support had the same disadvantage. Celites (Johns-Manville grades 545 and 560; L. Light & Co. Ltd.) and crushed Silocel C22 firebrick (L. Light & Co. Ltd.) were tried as supports, all deactivated with dimethyldichlorosilane by the method of SJÖVALL, MELONI AND TURNER[33]. Of these, C22 firebrick was best, both in terms of peak efficiencies and also because much higher ratios of stationary phase could be used without the packing losing its free flowing quality. Deactivation with hexamethyldisilazane[34], diphenyldichloro-silane, polyvinylpyrrolidone (PVP) and "Saran" were also tried, the last two using the method of VANDENHEUVEL, GARDINER AND HORNING[35]. PVP gave slightly higher peak efficiencies compared with the method of SJÖVALL *et al.*[33], but appeared to catalyse the decomposition of the serine derivative and probably the threonine derivative (see Table IV and Discussion). "Saran" was not very effective for deac-

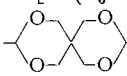
tivating the C22 firebrick. Diphenyldichlorosilane was as good as dimethyldichlorosilane, but less convenient in practice, since a toluene solution of phenol had to be used for reacting with residual chloro groups, and the excess phenol had to be washed out with toluene.

(*b*) *Preparation of the support.* Silocel C22 firebrick was crushed in a mortar, and graded dry using British Standard test sieves and a mechanical shaker (Endecotts (Filters) Ltd., Lombard Road, London, S.W. 19). The graded fractions were soaked in conc. HCl overnight and washed with acid until free of iron. After washing with distilled water until the washings were free of chloride, the support was dried in an oven at 100°. The individual acid-washed fractions were then re-graded before deactivation by the method of SJÖVALL *et al.*[33]. While peak efficiencies were improved by either acid washing or DDS deactivation, the highest efficiencies were obtained when both were carried out.

(*c*) *Stationary phases.* QF-1 (fluorosilicone fluid FS 1265, 10,000 cs) was obtained from Midland Silicones Ltd., Barry, Glamorgan. Other MS silicones (Midland Silicones Ltd.) were obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex. F-50 (methyl chlorophenyl silicone) and XE-60 (cyanoethyl silicone gum) were obtained from F & M Scientific Europa N.V., Leidsestraat 67, Amsterdam, M & B "Embaphase" (dimethyl polysiloxane) from May and Baker, Ltd., Dagenham, Essex, and SE 30 (dimethyl silicone elastomer E 301) from I.C.I. Ltd., Stevenston, Ayrshire. SG (stopcock grease) and HVG (high vacuum grease) (Edwards High Vacuum Ltd.) are generally available; HVG was purified by the method of NELSON AND MILUN[36]. BDA (butane-1,4-diol–adipic acid polyester) and BDS (butane-1,4-diol–succinic acid polyester) were prepared by the method of FARQUHAR, INSULL, ROSEN, STOFFEL AND AHRENS[37]. Hi-Eff-8B (cyclohexane dimethanol–succinic acid polyester) was obtained from Applied Science Laboratories Inc., P.O. Box 140, State College, Pa., U.S.A. DEGA (diethylene glycol–adipic acid polyester), NPGS (neopentyl glycol–succinic acid polyester), NPGG (neopentyl glycol–glutaric acid polyester) and NPG Seb (neopentyl glycol–sebacic acid polyester) were prepared by the method of FARQUHAR *et al.*[37]. PVP (polyvinylpyrrolidone) was obtained from British Drug Houses Ltd.; "Saran" (Saran resin F 220) from R. W. Greeff & Co. Ltd., 31 Gresham St., London E.C. 2. PEG-A (polyethylene glycol adipate) was obtained from W. G. Pye & Co. P.O. Box 60, Cambridge, and PPS (polypropylene sebacate) and EGS (ethylene glycol distearate) from Griffin & George Ltd. GMHS (glyceryl monohydroxystearate), GML (glyceryl monolaurate), GMO (glyceryl monooleate), GMR (glyceryl monoricinoleate), GMS (glyceryl monostearate), WOL (polyglycerol–polyricinoleic acid polyester), Admul 19 (polyglycerol ester of mixed fatty acids) and Admul S 57 (polyglycerol ester of hardened tallow fatty acids) were obtained from Advita Ltd., New Zealand House, Wellington Avenue, Walton-on-Thames. WOL and Admul S57 were purified by suspending in aqueous acetone and boiling with 1 g of Bio-deminrolit (Permutit Ltd.) per 2 g of polyglycerol ester. After filtration and washing the resin with aqueous acetone the solvent was removed from the combined filtrates on a rotary evaporator. The residues were dried in a vacuum desiccator. TWEEN 60 and 61 (polyoxyethylene Sorbitan monostearate), TWEEN 65 (polyoxyethylene Sorbitan tristearate), TWEEN 80 and 81 (polyoxyethylene Sorbitan monooleate), TWEEN 85 (polyoxyethylene Sorbitan trioleate), SPAN 20 (Sorbitan monolaurate), SPAN 40 (Sorbitan monopalmitate), SPAN 60 (Sorbitan monooleate)

and SPAN 85 (Sorbitan trioleate) were obtained from L. Light & Co. Ltd.

Acetyl SPAN 80 was prepared from SPAN 80 by leaving it for 72 h dissolved in 10 volumes of acetic anhydride. Excess of the anhydride was removed under vacuum and the residue was used as Acetyl SPAN 80.

U 1 (triphenyl germanium oxide), U 2 (tri-*n*-hexylgermanium oxide), U 3 $[\text{-Ge}(C_6H_5)\text{-}C_6H_4\text{-Ge(Me)}_2\text{-}C_6H_4]_n$, U 4 (dibutyltin sulphide), U 5 (tetra-*β*-cyanoethyltin), U 6 (dibutyltin dilaurate), U 7 (tetra-*n*-octyl-tin), U 8 $[Sn(C_6H_5)_2\text{-CH=CH-(CH}_2)_6\text{-CH=CH}]_n$, U 9 $[Sn(C_4H_9)_2\text{-CH=CH-(CH}_2)_5\text{-CH=CH}]_n$, U 10 $[Sn(C_3H_7)_2\text{-CH}_2\text{-CH(C}_6H_5)]_n$, U 11 $[Sn(C_6H_5)_2\text{-(CH}_2)_3\text{—O-CO-CH}_2\text{-O-CH}_2\text{COO-(CH}_2)_3]_n$ and U 12 $[Sn(C_6H_5)_2\text{-(CH}_2)_2$  $\text{-(CH}_2)_2\text{—}]_n$ were donated by Dr. J. W. MARSMAN of Utrecht University. Tetraphenyltin was obtained from Eastman Organic Chemicals (through Kodak Ltd., Kirkby, Liverpool) and TPMT (triphenyl-methyltin) was donated by Dr. G. A. LUIJTEN of Utrecht University. Methyl cyclohexyl titanate and triethanolamine titanate were donated by Mr. P. BROOKS of Griffin & George Ltd., triethanolamine titanate stearate was purchased from Griffin & George Ltd., and tetrastearyl titanate from K & K Chemicals Inc. (through Kodak Ltd.). DSPO (Duo-Seal pump oil, Pyror S.A., Geneva) was supplied through the courtesy of Camlab (Glass) Ltd. Cambridge. Ethanolamine adipate was prepared according to the method of FARQUHAR *et al.*[37]. EGCNSS-S(ethylene glycol succinate–cyanoethyl silicone copolymer) and EGSS-X (ethylene glycol succinate–methyl silicone copolymer) were obtained from Applied Science Laboratories Inc. KEL-F No. 90 grease (chloro-fluorocarbon, Minnesota Mining & Mfg. Co.) was obtained from British Drug Houses Ltd., Poole, Dorset. PEG 1540 and PEG 20M (polyethylene glycols) were obtained from Union Carbide Ltd. Grafton Street, London, W.1. PEG-T (TFA Carbowax PEG 20M) was made by treating 1 g PEG 20M dissolved in 20 ml trifluoroacetic acid with 5 ml of trifluoroacetic anhydride for 18 h at room temperature. Removal of excess reagents *in vacuo* gave 2.1 g of a viscous syrup, which was kept in a vacuum desiccator. TTP (tri-*o*-tolyl phosphate) was obtained from British Drug Houses Ltd. PEG-L (polyethylene glycol lauryl ether) was obtained from Honeywill & Stein Ltd., Mayfair Place, London W.1. Versamid 900 (polyamide from dimerised linoleic acid and ethylene diamine) was obtained from F & M Scientific Europa N.V., and TAS 10 (tetra-*o*-tolyl silicate) from Monsanto Chemicals Ltd., Victoria St., London S.W.1., was prepared from TAS 10A, a solution in decalin, by removing the solvent by vacuum distillation. TAS 190 (phenyl resorcinyl silicate), TAS 1,000 (tetraphenyl silicate) and HT-180S (phenyl-triphenoxy-silane) were obtained from Hygrotherm Engineering Ltd., Pebblecombe Research Station, Pebblecombe Hill, Dorking Road, Tadworth, Surrey.

(*d*) *Coating the support.* The correct weight of stationary phase was dissolved in 200 ml of a suitable solvent in a 500 ml round-bottomed flask with four dimples round the side. The correct weight of deactivated support was added, and the solvent removed under vacuum on a rotary evaporator (at low speed to avoid abrasion of the support particles).

(*e*) *Packing the columns.* Columns were cleaned by passing successive plugs of fine grade steel wool through them until the surface was burnished, and finishing off with successive plugs of cotton wool, first soaked in benzene and subsequently dry. Packings were poured continuously through a funnel into each arm of a column which

was kept vibrating by means of an electric motor. Final settling was achieved by manual tapping with a piece of wood. A plug of glass yarn (900-5/5 HT sewing thread, Fibreglass Ltd., 63, Piccadilly, London) was put at each end of the column, which was checked for leaks before insertion in the machine, and then conditioned for at least 12 h at the temperature of operation with a slow flow of nitrogen passing through it.

Preparation of the volatile derivatives

(*a*) *TFA amino acid methyl esters.* 2 mg of the amino acid was placed in a B 14 test tube with 2 ml methanol. The tube was kept in an oil bath at 70° and a continuous stream of dry HCl gas was passed through the alcohol through a Pasteur pipette for 20 min. The alcohol was evaporated using a rotary evaporator and a hot water bath. To the residue, 0.1 ml TFA anhydride was added, and the tube stoppered and left at room temperature for one hour. Excess of the anhydride was removed on the rotary evaporator at room temperature. The N-TFA amino acid methyl ester was taken up in 100 or 200 μl methyl ethyl ketone or nitromethane.

The preferred method was to treat the amino acid with 0.1 ml of TFA anhydride in a B14 test tube, and to leave the tube stoppered at room temperature for one hour. The excess anhydride was removed as before, and to the residue was added 1–2 ml of an ethereal solution of diazomethane (CARE: carcinogenic agent), prepared by the method of DE BOER AND BACKER[38]. After about 10 min, excess ethereal reagent was removed on a warm water bath before dissolving up the residue as above.

(*b*) *Ethyl, propyl and butyl esters.* The ethyl ester was made like the methyl ester, only at 80° instead of 70°, while 108° was used for all higher aliphatic alcohols.

(*c*) *Benzyl esters.* These were made using phenyldiazomethane prepared either by the method of YATES AND SHAPIRO[39] or of OVERBERGER AND ANSELME[40]. The benzyl esters were then treated with TFA anhydride as above.

(*d*) *Amyl esters.* The method of JOHNSON *et al.*[15] was tried, using HCl gas instead of HBr, and was found to give variable yields. Poor yields were obtained when a strongly acidic resin was used as catalyst[23]. The method used by ZOMZELY *et al.*[26] was tried with amyl alcohol and di-*n*-amyloxy-propane[41–43] but was not very successful. Finally the method outlined in (b) above was used, passing HCl gas at 108° for 25 min. The alcohol was removed on a rotary evaporator under vacuum (oil-pump), and the amino acid ester hydrochloride trifluoroacetylated as described above. The 99 % 1-pentanol used was obtained from Union Carbide Chemicals Co., Texas City, Texas.

RESULTS

General

The retention times of four amino acids as their N-acetyl and N-TFA ester derivatives are given in Table I on both a polar and a non-polar stationary phase. It can be seen that the retention time increases as the ester group becomes larger. Also, the non-polar QF-1 column shows shorter retention times than the polar PEG-A, whilst the N-acetyl derivatives are always much slower than the corresponding N-TFA derivatives[44,45]. It will be noted that glycine is disproportionately retarded on PEG-A relative to the other three amino acids. This anomaly has been repeatedly observed

TABLE I

RETENTION DATA FOR N-TFA AND N-ACETYL AMINO ACID ESTERS

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivatives is given in brackets.
Column temp. 150°. Flow rate 38 ml/min. 5% PEG-A and 5% QF-1 on HCl-washed, DDS-treated 150–200 mesh Silocel C22.

Amino acid ester	*5% PEG-A*	*5% QF-1*	*Amino acid ester*	*5% PEG-A*	*5% QF-1*
N-TFA Ala methyl	0.61	0.63	N-Acetyl Ala methyl	0.52	0.49
N-TFA Val methyl	0.63	0.70	N-Acetyl Val methyl	0.67	0.84
N-TFA Gly methyl	1.21	0.62	N-Acetyl Gly methyl	0.86	0.51
N-TFA Leu methyl	(10.6)	(7.9)	N-Acetyl Leu methyl	(66.7)	(29.3)
N-TFA Ala ethyl	0.67	0.53	N-Acetyl Ala ethyl	0.53	0.47
N-TFA Val ethyl	0.69	0.63	N-Acetyl Val ethyl	0.66	0.73
N-TFA Gly ethyl	1.28	0.63	N-Acetyl Gly ethyl	0.90	0.55
N-TFA Leu ethyl	(10.6)	(9.4)	N-Acetyl Leu ethyl	(70.0)	(31.7)
N-TFA Ala amyl	0.62	0.51			
N-TFA Val amyl	0.60	0.73			
N-TFA Gly amyl	1.39	0.59			
N-TFA Leu amyl	(31.4)	(26.6)			

when comparing relative retention times for glycine on polar and non-polar phases (see also DORLET[45]). It may be that the active hydrogen atoms on the α-carbon of glycine interact with the groupings that confer polar character on the stationary phase. Since the TFA group is more electronegative than the acetyl group one might expect this effect to be accentuated with the N-TFA derivatives of glycine, and indeed Table I shows that for these compounds the increased activity of the hydrogens on the α-carbon is reflected in the increased relative retention times on PEG-A.

The peaks obtained on chromatography of the N-TFA amino acid methyl esters were always satisfactory, and since these derivatives are the most volatile of those shown in Table I they afforded the most likely chance for working at moderate temperatures, which was of potential value particularly for the derivatives of the less volatile amino acids. However, a study of quantitative yields in the preparation of the derivatives of the more volatile amino acids revealed such wide variation that it was evident that extensive losses were occurring, which could be traced to the process of evaporating the excess ethereal diazomethane after esterification of the N-TFA amino acid. This occurred with the derivatives of alanine, valine, glycine, leucine and isoleucine. In the case of the alanine derivative, sublimation was observed as it was being evaporated to dryness in a rotary evaporator under vacuum. The high vapour pressure of N-TFA amino acid esters was used by WEYGAND and his school[20,46] to achieve partial resolution into groups by vacuum sublimation.

Table II shows the results of an experiment in which the volatility of the N-TFA esters of alanine are compared. Known amounts of the derivatives were kept in a gentle stream of gas at room temperature for varying times, and the percentage of the starting material that remained was determined by gas chromatography. It is clear that losses occur under these conditions, and indeed, by using a more vigorous stream of gas, N-TFA alanine methyl ester may be evaporated completely, and extensive losses occur even with the corresponding derivatives of glycine and leucine.

TABLE II

LOSSES OF AMINO ACID DERIVATIVES OWING TO VOLATILITY

Pretreatment: Argon 250 ml/min, diameter of jet 1.1 mm, distance from ester 3 cm in a tube 1.0 cm I.D.
Column temp. 108°. Flow rate 38 ml/min. 1 % PEG-A on 100–120 mesh HCl-washed, DDS-treated Celite 560.

N-TFA alanine ester	*Percentage recovery after exposure to stream of gas*		*Retention time (min)*
	5 min	*20 min*	
Methyl	66	21	3.1
Ethyl	61	6	3.2
n-Propyl	100	61	4.9
n-Butyl	96	73	7.0
n-Amyl	99	93	11.5

Only the *n*-amyl esters give a reasonable assurance that losses will not occur during handling. It was found to be safe to use a rotary evaporator for the removal of solvents.

The cyclohexyl esters of the N-TFA amino acids always gave multiple peaks for each amino acid. The benzyl esters, prepared by either of the two methods quoted[39,40] contained, in addition to the expected derivative, bibenzyl and another unidentified volatile compound. The production of more than one peak from each amino acid is confusing, and therefore these derivatives were not used.

By restricting ourselves to the TFA amino acid amyl esters, and by removing excess solvents on the rotary evaporator no losses by evaporation were detected during the preparation of the volatile derivatives.

NOTES ON TABLES III TO VII

Table III

The relative retention times for the phases in this table are broadly similar in pattern, although a number of sub-groups may be recognised: those like SE-30 (MS 200, SG, MS Antifoam A) and those like MS 550 and F-50. The pattern of MS 710 is more like that of the silicates TAS 10 and HT-180S in Table VII.

Using the relative retention time for the glycine derivatives as an approximate guide to the polarity of a phase, XE-60 may be seen to be the most polar, and SE-30 the least polar of the silicones. By this criterion many phases mentioned in later tables are more polar than XE-60. The pattern on XE-60 resembles those for the polyesters in Table IV. Serine and threonine have very similar retention times on MS 115, MS 200, M & B and F-50. Leucine and isoleucine run together on many of these silicones. The relative retention times of the aromatic compounds on QF-1 are the lowest we have found.

Efficiencies obtained with SG, HVG and MS Antifoam A were low.

Table IV

The patterns of relative retention times on these phases varied widely, and no

TABLE III

RELATIVE RETENTION DATA FOR SILICONE TYPE STATIONARY PHASES

The figures are retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
MS 115	5	130	38	0.41	0.69	0.47	1.00	(47.8)	0.59	0.65	0.66	1.33	0.9
MS 200	5	140	38	0.44	0.73	0.49	1.01	(16.6)	0.57	0.61	0.66	1.23	1.4
MS 550	2	141	38	0.45	0.67	0.57	1.04	(16.9)	0.57	0.68	0.86	1.67	1.2
MS 710	5	159	45	0.48	0.70	0.62	1.00	(10.5)	0.46	0.60	1.21	n.r.	1.7
M & B	5	130	40	0.41	0.68	0.49	1.03	(17.8)	0.63	0.69	0.69	1.32	1.0
F-50	5	150	40	0.48	0.51	0.78	1.15	(13.2)	0.58	0.61	0.85	n.r.	0.9
QF-1	5	150	38	0.51	0.73	0.59	0.60	(26.6)	0.88	1.09	0.30	0.45	0.7
SE-30	5	132	38	0.39	0.68	0.46	1.01	(37.0)	0.54	0.64	0.64	1.23	1.1
XE-60	5	150	38	0.53	0.61	0.92	0.82	(26.5)	0.81	1.47	0.37	0.67	1.1
S.G.	2	132	38	0.48	0.67	0.55	1.17	(10.8)	0.62	0.74	0.66	1.20	2.4
H.V.G.	2	130	38	0.52	0.72	0.57	1.00	(11.5)	0.63	0.71	0.71	1.19	1.7
MS Anti-foam A.	2	132	38	0.44	0.69	0.53	1.11	(9.0)	0.63	0.72	0.59	1.11	2.0

n.r. = compound not run.

TABLE IV

RELATIVE RETENTION DATA FOR POLYESTER-TYPE PHASES

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
BDA	5	150	38	0.59	0.59	1.22	0.76*	(30.7)	0.62	1.32	0.89	1.49	0.8
BDS	5	160	40	0.69	0.63	1.42	0.77*	(14.1)	0.65	—	1.11	n.r.	1.0
Hi-Eff-8B	5	150	38	0.58	0.58	1.17	0.77*	(24.5)	—	—	1.11	1.77	0.8
DEGA	2	145	38	0.60	0.60	1.19	0.79	(15.2)	0.79	1.45	n.r.	1.28	1.0
NPGS	10	152	50	0.57	0.57	0.85	0.65*	(66.0)	0.60	1.14	0.53	n.r.	0.7
NPGS + PVP	9 / 1	150	38	0.59	0.59	1.17	0.77*	(63.5)	0.80	—	0.61	1.04	0.6
NPGG	5	150	38	0.57	0.57	1.11	0.78	(31.4)	0.68	—	0.67	1.13	1.4
NPGSeb	5	150	38	0.50	0.57	0.90	0.78*	(33.5)	0.58	—	0.69	1.28	0.8
PEG-A	5	150	38	0.62	0.61	1.39	0.74*	(31.4)	0.70	1.43	0.96	1.52	0.8
PPS	5	150	38	0.47	0.56	0.89	0.78*	(48.5)	—	—	0.68	1.21	0.7

* Shows partial resolution of alloiso- and isoleucine.

n.r. = compound not run.

— = compound applied but gave no peak.

definite regularities are immediately apparent. These phases, in general, exhibit high efficiencies (except NPGG) and symmetrical peaks, and some of them are able to effect a partial resolution of the isoleucine peak into alloisoleucine and isoleucine, which usually emerge in that order. No phase was found which resolved these two completely.

If one applies the criterion of polarity mentioned before (relative retention time for the glycine derivative) these polyesters are generally more polar than the silicones. BDS is the most polar, and NPGS the least polar.

Table V

The behaviour of the derivatives of serine and threonine on columns of this type is significant. Most of these surface active agents appear to be active also as catalysts in the decomposition of the derivatives of serine and threonine. The extent of the decomposition is a function of the time the derivatives are in contact with the phase, so that serine, with longer retention times, is always more affected than threonine.

Detailed studies with EGS have shown that when conditions are used where retention times are short (low proportion of stationary phase, higher temperatures, rapid flow rates) both derivatives emerge from the column, but when retention times are progressively increased first the serine and then the threonine peaks fail to come off. This is discussed more fully later.

Many of these phases are able to effect partial resolution of the isoleucine peak. TWEENS 20, 40, 60, 80 and acetyl SPAN 80 are highly polar (see glycine).

Studies with the SPAN and TWEEN series might offer an opportunity for analysing the interaction of compounds with stationary phases which vary in a controlled manner to see whether any underlying regularities or generalisations can be detected. Acetylating SPAN 80 shows the effect of a relatively simple chemical modification on the polarity of a stationary phase.

Table VI

With the exception of U 4, U 6 and TPMT, efficiencies of the peaks obtained on these organometallic phases were low. As one might expect from the variety of structure that is to be found within this group, there are also wide variations in polarity. The most polar was U 12, where no peak for glycine was observed after 60 min, and U 5 also showed high polarity. Threonine and serine derivatives were also affected in many cases.

A number of other organometallic compounds were investigated in addition to those quoted. A tin compound designated U 9 $[Sn(C_4H_9)_2\text{-}CH{=}CH\text{-}(CH_2)_5\text{-}CH{=}CH\text{-}]_n$ had the property of catalysing the complete destruction of all the amino acid derivatives, although biphenyl and bibenzyl emerged normally. The same behaviour was noted with the four titanium compounds methyl cyclohexyl titanate, tetrastearyl titanate, triethanolamine titanate and triethanolamine titanate stearate.

Table VII

Although it is clearly unprofitable to expect any regularities where there are no chemical relationships, certain affinities may be recognised. Thus there is a resemblance between DSPO and TAS 10 and Kel-F has a resemblance to silicone MS 550 in Table III.

TABLE V

RELATIVE RETENTION DATA FOR SURFACTANTS

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w liquid–solid*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
EGS	5	150	38	0.40	0.62	0.59	0.88	(38.8)	—	—	0.91	1.76	0.8
GMHS	2	152	38	0.49	0.63	0.70	0.93	(7.0)	0.56	0.83	0.84	1.54	2.0
GML	2	152	38	0.49	0.68	0.65	0.93*	(12.0)	0.53	0.76	0.83	1.49	0.8
GMO	2	152	38	0.45	0.66	0.70	0.88	(12.0)	0.55	0.83	0.85	1.57	0.8
GMR	5	155	38	0.47	0.61	0.84	0.89*	(31.0)	0.51	—	n.r.	1.62	0.5
GMS	5	150	38	0.40	0.64	0.64	0.88*	(56.5)	0.49	—	0.85	1.62	0.4
WOL	2	130	38	0.41	0.56	0.71	0.80*	(55.0)	0.49	0.84	0.75	1.50	0.5
Admul 19	2	130	38	0.42	0.57	0.76	0.90	(37.7)	0.48	—	0.71	1.37	0.8
Admul S57	2	150	38	0.46	0.61	0.73	0.91	(14.0)	0.56	—	0.85	1.61	1.2
TWEEN 20	5	150	38	0.61	0.56	1.56	0.74*	(28.5)	—	—	1.12	1.83	0.5
TWEEN 21	5	149	38	0.58	0.58	1.19	0.77*	(49.2)	—	—	0.73	1.27	0.9
TWEEN 40	5	150	38	0.60	0.57	1.53	0.75*	(30.7)	—	—	1.04	1.72	0.9
TWEEN 60	5	150	38	0.61	0.56	1.45	0.77*	(31.0)	—	—	1.00	1.69	0.8
TWEEN 61	5	150	38	0.49	0.58	0.99	0.79*	(39.0)	0.46	—	0.87	1.52	0.6
TWEEN 65	5	150	38	0.52	0.58	1.10	0.79*	(38.6)	0.45	—	0.69	1.24	0.7
TWEEN 80	2	150	38	0.64	0.63	1.50	0.78	(9.9)	—	—	1.07	1.68	0.8
TWEEN 81	5	150	38	0.49	0.56	1.02	0.78	(20.9)	0.53	—	0.86	1.50	1.2
TWEEN 85	5	150	50	0.53	0.60	1.16	0.80*	(34.7)	0.68	—	0.97	1.70	0.6
SPAN 20	5	150	38	0.47	0.60	0.85	0.85	(30.7)	—	—	0.73	1.35	0.7
SPAN 40	5	150	38	0.47	0.61	0.77	0.88*	(21.0)	—	—	0.84	1.57	0.6
SPAN 60	2	150	55	0.50	0.64	0.80	0.91	(8.2)	0.50	—	0.88	n.r.	1.0
SPAN 65	2	150	53	0.51	0.58	0.72	0.92	(6.5)	0.46	0.66	0.91	n.r.	1.4
SPAN 80	5	140	38	0.44	0.58	0.73	0.85*	(49.7)	—	—	0.78	1.50	0.6
SPAN 85	5	150	38	0.49	0.60	0.88	0.84	(20.3)	0.48	—	0.87	1.53	0.8
Ac SPAN 80	5	150	60	0.58	0.57	1.40	0.74*	(18.2)	0.49	—	1.05	n.r.	1.0

* Shows partial resolution of alloiso- and isoleucine.
n.r. = compound not run.
— = compound applied but gave no peak.

TABLE VI

RETENTION DATA FOR METALLO-ORGANIC STATIONARY PHASES

The figures are the retention times relative to the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Liquid phase	*% w/w phase–support*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
U1	2	150	38	0.62	0.74	0.72	1.02	(7.4)	0.61	—	1.39	2.34	2.1
U2	2	152	38	0.49	0.74	0.57	1.07	(6.1)	0.54	—	1.11	2.13	1.4
U4	2	131	38	0.46	0.63	0.76	1.09	(19.7)	0.53	0.76	1.19	2.62	0.7
U5	5	150	40	0.73	0.66	1.54	0.79	(14.3)	—	—	0.85	1.20	1.2
U6	2	145	38	0.42	0.60	0.67	0.91	(13.0)	—	—	n.r.	1.71	0.9
U7	2	131	38	0.57	0.71	0.46	1.00	(24.9)	0.45	0.50	1.09	2.27	2.2
U8	2	123	38	0.40	0.62	0.63	1.00	(19.5)	—	—	1.70	3.49	1.9
U10	2	152	38	0.56	0.75	0.60	0.98	(4.8)	—	—	1.04	1.81	
U11	5	151	38	0.61	0.61	1.31	0.79	(12.4)	—	—	0.86	1.15	1.5
U12	5	150	38	0.53	0.65	—	0.89	(13.4)	—	—	1.46	2.34	2.1
TPMT	10	153	40	0.43	0.62	0.67	1.00	(31.5)	—	—	1.48	n.r.	0.5

n.r. = compound not run.
— = compound applied but gave no peak.

TABLE VII

RETENTION DATA FOR MISCELLANEOUS TYPES OF STATIONARY PHASES

The figures are the retention times relative to that for the leucine derivative taken as 1.00. The actual retention time in minutes for this derivative is given in brackets.

Stationary phase	*% w/w phase–support*	*Column temp. (°C)*	*Gas flow (ml/min)*	*N-TFA amino acid n-amyl ester*					*O,N-DiTFA n-amyl ester*		*Biphenyl*	*Bibenzyl*	*H.E.T.P. (mm) Leu peak*
				Ala	*Val*	*Gly*	*Ileu*	*Leu*	*Thr*	*Ser*			
DSPO	5	151	30	0.39	0.45	0.68	1.05	(32.0)	0.42	0.47	1.38	2.75	0.6
EAA	5	153	35	0.80	0.55	2.09	0.69	(6.5)	0.61	—	0.91	n.r.	2.6
EGCNSS-S	5	141	38	0.67	0.59	1.54	0.76	(20.8)	0.74	—	0.76	1.18	1.4
EGSS-X	5	158	60	0.84	0.68	1.66	0.82	(4.4)	0.66	—	1.25	n.r.	2.3
KEL-F 90	5	150	40	0.47	0.58	0.75	1.03	(7.7)	0.69	0.78	0.73	n.r.	2.0
PEG 1540	1	130	60	0.63	0.44	1.81	0.67	(7.9)	—	—	0.85	1.32	1.8
PEG 20M	10	201	40	0.83	0.70	1.76	0.83	(6.3)	—	—	1.92	2.63	1.2
PEG-T	5	152	55	0.73	0.56	1.97	0.73	(4.2)	—	—	1.16	1.77	1.0
PEG-L	5	150	38	0.64	0.56	1.64	0.74*	(25.0)	—	—	1.12	1.84	1.0
TTP	5	152	60	0.47	0.51	0.61	0.70*	(86.5)	1.01	—	0.99	n.r.	0.7
Versamid 900	5	170	60	0.58	0.58	1.08	0.80	(10.0)	1.10	—	n.r.	1.50	1.7
TAS 10	5	153	35	0.58	0.72	0.80	0.98	(7.1)	0.44	0.62	1.95	n.r.	2.9
TAS 190	5	153	26	0.51	0.65	0.77	1.00	(11.0)	0.45	0.55	n.r.	n.r.	1.8
TAS 1,000	5	144	50	0.48	0.66	0.71	0.95	(8.9)	0.41	0.54	1.49	n.r.	0.8
HT-180S	5	141	37	0.45	0.66	0.68	0.96	(24.7)	0.40	0.54	1.66	3.36	1.9

* Shows partial resolution of alloiso- and isoleucine.

n.r. = compound not run.

— = compound run but gave no peak.

The two Carbowax phases are similar, and show a resemblance to acetylated SPAN 80, and to some of the TWEENS (Table V). Other members of this group are PEG-T, PEG-L, EAA and the silicone-polyesters EGCNSS-S and EGSSX.

Just as the polarity of SPAN 80 was affected by acetylation so that of PEG 20M is affected by trifluoroacetylation, but to a lesser degree. The O-TFA group is not so stable as the N-TFA group, and it was found that the PEG-T deteriorated in use.

The figures quoted for TAS 10 are of interest since with this phase threonine emerges first, very much earlier than alanine. The chemically related aromatic silicates (TAS 190, TAS 1,000 and HT-180S), and TWEEN 65, SPAN 65 and Ac SPAN 80 also show this effect but to a lesser extent. The resolution of the other amino acid derivatives is quite good with TAS 10, but all the aromatic silicates appear to have insufficient stability and are too volatile for routine use as stationary phases above 120°.

DISCUSSION

As has already been mentioned earlier, serine and threonine derivatives are subject to decomposition catalysed by some of the stationary phases, due probably to the labile nature of the O-TFA group, and evidence has been obtained indicating that the product of such a reaction is the mono-substituted N-TFA derivative. This is still volatile and its retention time is about double that of the O,N-di-TFA derivative on XE-60. However, owing perhaps to the possibility of N → O acyl shifts (WEYGAND AND RINNO[47]), these N-mono-TFA derivatives are also heat-labile, so that after destruction no volatile products are recovered. Polar polyesters of the surfactant type appear to be the most active catalysts. It is in fact possible to obtain peaks of the O,N-di-TFA derivatives of serine and threonine from even the most catalytically active phases by using higher temperatures and fast flow rates, *i.e.* conditions where the time that the derivative is in contact with the phase is minimised. This phenomenon has practical significance in quantitative studies.

One factor which must be noted when studying the retention data of Tables III-VII is that in many cases retention times were obtained with individual amino acid derivatives, or pairs of derivatives. However, when applied in the full seven-component mixture retention times sometimes change from the figures quoted in the tables, so that some peaks coalesce, and the full resolution is not attained.

In most cases attempts have been made to optimise resolutions by variations not only of the operating temperatures and flow rates, but also by varying the ratio of the amount of stationary phase to support. It has been found, in agreement with other workers, that at higher values of this ratio higher peak efficiencies are obtained. There seems to be, for each phase, a critical value for the ratio of phase to support below which the H.E.T.P. rises steeply (peaks become broad): thus for XE-60 this is about 5 %, while for WOL it is 2 % (both on C22 firebrick).

No single column packing has been found which allows the seven most volatile amino acids to be separated completely under the isothermal conditions used. However, some useful separations can be recommended. Fig. 1 shows the resolution of alanine, threonine, valine, glycine, isoleucine and leucine, on 2 % WOL at 130°. The isoleucine peak is not quite resolved into alloisoleucine and isoleucine; the serine peak, however, comes directly on top of these two. Fig. 2 shows the resolution of

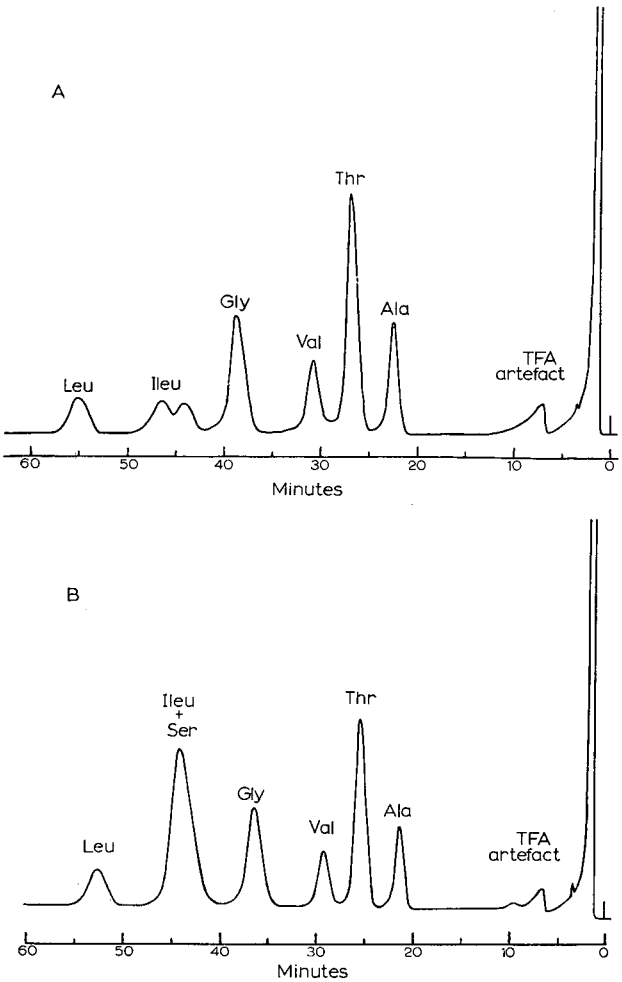


Fig. 1. Separation of N-TFA amino acid *n*-amyl esters (O,N-di-TFA derivatives of serine and threonine) on WOL. Conditions: 2% WOL on 150–200 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 130°; 1.45 kg/sq.cm; 40 ml N_2/min. A: without serine; B: with serine.

alanine, valine, glycine, isoleucine and leucine on 5% SPAN 80 at 150°. Threonine and serine are completely decomposed under these conditions unless present in high concentration. The isoleucine also appears in the partially resolved form. Fig. 4 shows good resolution of alanine, valine, isoleucine, glycine, leucine and serine on 10% XE-60 at 140°. Threonine here coincides with isoleucine. Isoleucine is not split on this phase, although the alloisoleucine forms a shoulder on the peak.

A study of the retention data might suggest that it would be possible to achieve better resolutions by using columns in which more than one phase is present, either by putting lengths of different packings in the columns, by blending mixtures of packings, or by preparing packings in which the two (or more) different phases are applied to the support dissolved in the same solvent. All these methods of using

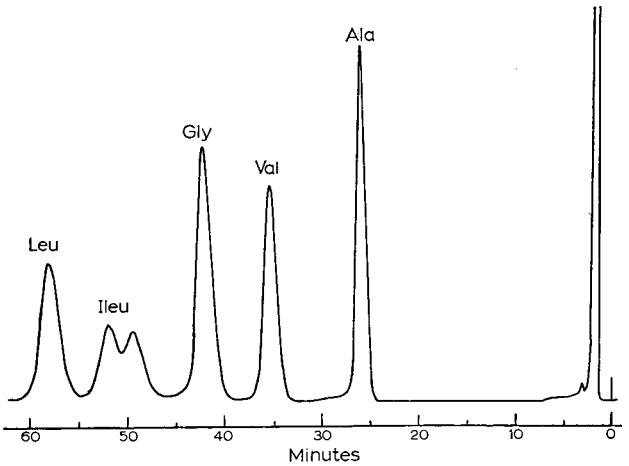


Fig. 2. Separation of N-TFA amino acid *n*-amyl esters on SPAN 80. Conditions: 5% SPAN 80 on 120–150 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 152°; 1.05 kg/sq.cm; 30 ml N_2/min.

mixed phases were in fact tried out, and it was found that the results were equivalent to what one would expect if the retention and other properties (*e.g.* efficiencies) behaved in an additive manner. However, it was not readily possible to find a combination capable of achieving a perfect resolution of the seven-component mixture, and this approach is being explored further.

For a purely qualitative separation it is not necessary to insist on resolution of such high quality, but if quantitative results are ultimately to be achieved, it is necessary for satisfactory integration that each peak should return to a stable baseline

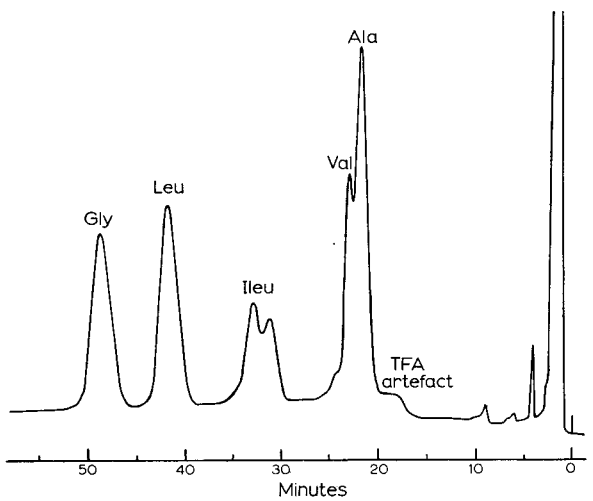


Fig. 3. Separation of N-TFA amino acid *n*-amyl esters on TWEEN 85. Conditions: 5% TWEEN 85 on 60–85 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 140°; 0.62 kg/sq.cm; 38 ml N_2/min.

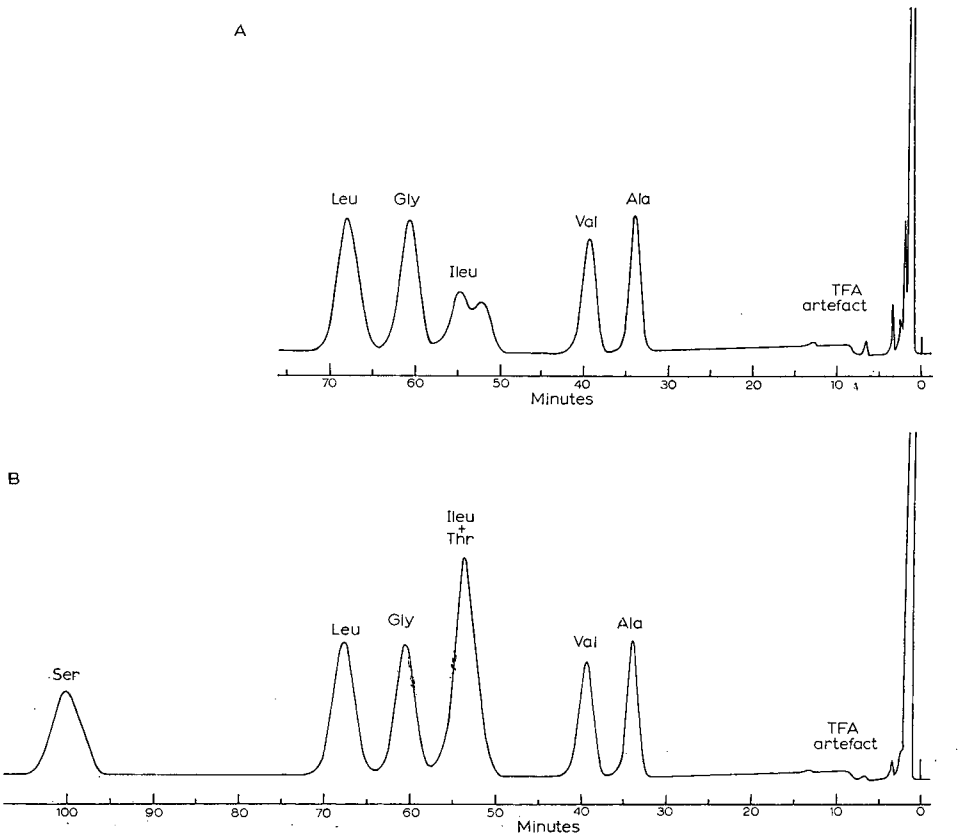


Fig. 4. Separation of N-TFA amino acid *n*-amyl esters (O,N-di-TFA derivatives of serine and threonine) on XE-60. Conditions: 10% XE-60 on 60–85 mesh, HCl-washed, DDS-deactivated Silocel C22 firebrick; 140°; 0.78 kg/sq.cm; 50 ml N_2/h. A: without serine and threonine; B: with serine and threonine.

before the emergence of the following peak. From the figures shown it is evident that for the derivatives used a quantitative estimation of all these seven amino acids cannot be obtained with a single run. However, it is possible to do so from runs on two different columns, by a process of subtraction. The problem therefore resolves itself into choosing suitable stationary phases for either threonine or isoleucine. For isoleucine, in addition to the SPAN 80 column in Fig. 2 one can also use TWEEN 85 (Fig. 3), but since alanine and valine are not resolved one looses some of the advantage of obtaining duplicate results.

The separations in Figs. 1–4 show rather slower runs than could be achieved, but under these conditions one obtains peaks with large areas, and therefore high counts on the integrator, which is of great potential advantage for accurate quantitative work.

The peak labelled "TFA artefact" is due to excess trifluoroacetic acid, and is only found when the last traces have not been removed after trifluoroacetylation, so that its appearance is sporadic. It has been included for illustrative purposes.

In practice we prefer to base quantitative work on XE-60 (Fig. 4), because of its high thermal stability, and to derive the isoleucine figures from SPAN 80, for in this way duplicate results are also obtained for alanine, valine, glycine, and leucine, while the use of these two columns aids unequivocal identification of the amino acids.

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SUMMARY

Methods are described for the preparation of volatile derivatives of amino acids, which permits analysis by gas chromatography. Alanine, glycine, isoleucine, leucine, serine, threonine and valine were converted to the trifluoroacetyl amino acid *n*-amyl esters, and the separation of these seven compounds was investigated on a wide variety of stationary phases. No single phase capable of achieving complete and unequivocal resolution was found, but the separation necessary for quantitative work was obtained by the use of two columns packed with different phases.

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GAS-CHROMATOGRAPHIC ANALYSIS OF POLYNUCLEAR ARENES

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INTRODUCTION

The analysis of polynuclear arenes is difficult because it usually involves the separation of the arenes from adventitious matter followed by partial or complete (liquid phase) chromatographic resolution into the individual compounds[1–11]. The identity of the "isolated" compound has invariably been deduced from spectroscopic data, usually from the ultra-violet absorption spectrum. This sequence is extremely time-consuming and requires very careful work. Moreover, the quantitative nature of the analysis is suspect since an isotope experiment has shown that losses can be as high as 50 %[8].

The methods referred to above were developed for analysing mixtures which contain predominantly the unsubstituted polynuclear arenes; they are not appropriate to systematic investigation of the alkyl derivatives which are of interest in the study of carcinogenesis[12–14] and of low-temperature pyrolysis.

The gas-chromatographic analysis of polynuclear arenes has been described by a number of workers, including DUPIRE[15–18], who chromatographed certain of the polynuclear hydrocarbons during work on the constitution of coal tar. He used a thermal-conductivity detector and worked at temperatures which cause substrate bleeding (ionization detector). CARUGNO AND GIOVANNOZZI-SERMANNI[19] analysed some of the polynuclear arenes with a low-resolution system having a detector of limited sensitivity (copper oxide combustion–thermal conductivity). GUDZINOWICZ AND SMITH[20] chromatographed triphenylene, picene and coronene. Sensitivity and resolution were low. FERRERO[21,22] analysed polynuclear arenes in coal tar. He used a thermal-conductivity detector and collected fractions for subsequent study. WOOD[23] also has analysed a coal tar–polynuclear arenes mixture using an emissivity detector. SAUERLAND[24,25] employed programmed-temperature chromatography in a study of the polynuclear arenes from coal tar fractions, using a thermal-conductivity detector and columns of low efficiency. FARRAND[26] attempted to analyse a coal tar polynuclear arene fraction using columns of low efficiency and a thermal-conductivity detector. LIJINSKY *et al.*[27] and LIJINSKY AND MASON[28] chromatographed polynuclear arenes on a glass-bead column using an ionization (argon?) detector. The column efficiency was low.

These methods are not suited to the *low-level analysis* of the complex mixtures of polynuclear arenes generally encountered in pollution studies and in other cases where the sample is limited in size.

During the analysis of a low-temperature pyrolysate[29] (from cholesterol) the

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present author found it necessary to determine methylchrysenes and alkylphenanthrenes in the presence of the parent compounds. Gas chromatography proved to be the most satisfactory of the methods tried; the technique has now been in use for some three years and has been extended to the analysis of higher systems.

EXPERIMENTAL

A laboratory-built instrument (with flame ionization detector and column outlet splitter) was used for the packed-column study, and a Perkin-Elmer model 154 chromatograph for the capillary column analyses. The injection block heater of the latter was disconnected (since it operated at pyrolytic temperatures) and replaced by an independently controlled heater.

The packed columns were of stainless steel, type 304, non-passivated, 4 mm and 2 mm I.D. The columns were helical, of 8 cm diameter.

The stationary phases were SE-30 and SE-52 (10 % by weight on the support) and Q-F1 (2.5 % by weight on the support). The support was of Chromosorb W, acid-washed, 60–80 mesh.

The fraction collectors were Pyrex tubes (10 cm × 2 mm, and 10 cm × 1 mm).

The capillary columns were of stainless steel, type 347, 0.5 mm and 1.0 mm I.D., coated with SE-30 from 5 % solution in chloroform.

For sample introduction a toluene solution (0.1–2.0 %) was used, injected with a micro-syringe.

RESULTS

Packed columns

Monel tubing was found to exhibit catalytic effects toward a number of hydroaromatic compounds, and copper was adsorptive for the polynuclear arenes. Stainless steel did not show these effects.

The tetracyclic and higher polynuclear arenes were adsorbed, to a slight degree, by the support; this did not produce tailing at the 10^{-6} g level. However, chrysene and benz(*a*)anthracene, while giving acceptably symmetrical peaks, were detectable (in the ultra-violet spectrum of a collected fraction) subsequent to the peak. The penta- and hexa-cyclic compounds, also, gave good peaks; the adsorbed material was, however, eluted only by displacement (by a similar or more highly condensed compound). The eluted compound was then seen in the ultra-violet spectrum of the displacing compound. The indicated adsorption level was 10^{-9} g/g support, which is of little significance in the analysis of samples of the order of 10^{-6} g.

Glass-bead packings proved too inefficient (HETP ~ 4 mm) for the analysis, although adding finely divided kieselguhr[30] to the packing resulted in an increase in the column efficiency (to an HETP of 1.4 mm). The necessarily high inlet pressures limited the length, and hence the efficiency, of the column; the maximum practical efficiency was some four times less than that of the Chromosorb-packed column. Adsorption (see above) was not detectable in an SE-30 glass-bead-in-stainless-steel column.

A loss of efficiency occurred when a newly conditioned (helical) column was allowed to cool (from an HETP of 0.8 to 1.2–1.4 mm for a 10 m SE-30 column cooling

TABLE I

RETENTION DATA[a] OF ARENES ON SE-30

Compound[b]	*Column temperature* (°C)		
	260		*300*
Fluorene	28		32.6
2-Methylfluorene	36.6		40.7
Anthracene	45.7		50
Phenanthrene	45.3		50
3-Methylphenanthrene	56		61.4
1-Methylphenanthrene	62.5		66.3
4,5-Methylenephenanthrene	—		67.1
9-Methylanthracene	68.1		70.9
1-Ethylphenanthrene	75.1		76.4
Fluoranthrene	87.4		88.3
Pyrene	100[c]		100
2-Methylpyrene	129		122
4-Methylpyrene	138		131
1-Methylpyrene	140		133
Benzo(*a*)fluorene	121		116
Benzo(*b*)fluorene	125		119
Benzo(*c*)fluorene	126		119
16,17-Dihydro-15H-cyclopenta(*a*)phenanthrene	134		127
17-Methyl-16,17-dihydro-15H-cyclopenta(*a*)phenanthrene	146		135
17-Methyl-15H-cyclopenta(*a*)phenanthrene	176		161
Benzo(*g,h,i*)fluoranthene	184		168
Triphenylene	207		182
Chrysene	208		183
Benz(*a*)anthracene	207		183
Naphthacene	221		195
2-Methylchrysene	272		233
3-Methylchrysene	268		231
4-Methylchrysene	279	282[d]	236
6-Methylchrysene	283		—
1-Methylchrysene	289		243
1-Methylbenz(*a*)anthracene		264[d]	—
9-Methylbenz(*a*)anthracene		272[d]	—
10-Methylbenz(*a*)anthracene		247[d]	—
3-Methylbenz(*a*)anthracene		272[d]	—
4-Methylbenz(*a*)anthracene		278[d]	—
7-Methylbenz(*a*)anthracene	302	302[d]	249
Benzo(*b*)fluoranthene	401		—
Benzo(*k*)fluoranthene	404		—
Benzo(*e*)pyrene	458		364
Benzo(*a*)pyrene	472		374
Perylene	492		385
3-Methylcholanthrene	585		439
Indeno(1,2,3-*c,d*)fluoranthene[e]	766		—
Indeno(1,2,3-*c,d*)pyrene[e]	875		—
Dibenz(*a,h*)anthracene	896		632
Benzo(*c*)tetraphene	927		654
Picene	931		663
Benzo(*g,h,i*)perylene	988		711
Anthanthrene	1050		753
Coronene	—		1350

[a] Relative to pyrene = 100; 10 m and 2 m columns.
[b] The nomenclature adopted for this paper is that recommended by I.U.P.A.C. (1957).
[c] Approximately 20 min.
[d] Extrapolated values.
[e] Identity based on ultraviolet spectrum of relevant fraction.

from 300° to ambient temperature). This effect, which was not cumulative, was not observed during subsequent heating cycles, and in straight or U-form columns was not significant. (It is considered that this loss of efficiency is due to mechanical stress applied to the packing during the coiling process.)

The stationary phases SE-30 and SE-52 were used up to 300°, which was found to be the upper limit for high-sensitivity analysis with the flame-ionization detector. The stationary phase Q-F1 was used up to 225°.

Retention data for the packed columns are given in Tables I–IV.

TABLE II

RETENTION DATA* OF ARENES ON SE-52

Compound	*Column temperature (°C)*	
	260	*300*
Fluorene	25	30
2-Methylfluorene	33	38
Phenanthrene	43	48
3-Methylphenanthrene	56	59
1-Ethylphenanthrene	73	74
Fluoranthene	87	87
Pyrene	100	100
1-Methylpyrene	135	131
Benzo(*b*)fluorene	125	—
Benzo(*c*)fluorene	125	—
17-Methyl-16,17-dihydro-15H-cyclopenta(*a*)phenanthrene	146	134
Triphenylene	—	189
Chrysene	216	190
Benz(*a*)anthracene	209	186
Naphthacene	230	203
2-Methylchrysene	276	240
3-Methylchrysene	276	240
4-Methylchrysene	292	245
6-Methylchrysene	290	—
1-Methylchrysene	300	252
7-Methylbenz(*a*)anthracene	315	262
Benzo(*e*)pyrene	494	—
Benzo(*a*)pyrene	512	—
Perylene	538	—

* Relative to pyrene = 100.

Capillary columns

The minimum tube diameter of 0.5 mm was dictated by the high viscosity of solutions (chloroform) of the phase SE-30.

The tetra- and penta-cyclic arenes were adsorbed (tailing) to a significant degree but this effect decreased when the film thickness was increased (by coating from a 5 % instead of a 1 % solution). As a result, the resolving power of the column for *n*-alkanes was decreased (from 1.0 to 0.78, HETP of 1.2 mm and 2.2 mm) whilst that for the arenes was increased (from 0.50 to 0.70 relative to the above figures).

Optimum resolution was attained for samples (on column) of $2 \cdot 10^{-7}$ to 10^{-6} g.

The columns have been used for prolonged periods (more than 3 months) at 260° without loss of efficiency.

The retention data for the capillary column are given in Table V.

TABLE III

RETENTION DATA* OF ARENES ON Q-F1**

Compound	*Column temperature (°C)*	
	200	*220*
Fluorene	16	—
2-Methylfluorene	22	—
Anthracene	35	—
Phenanthrene	34	—
3-Methylphenanthrene	48	—
4,5-Methylenephenanthrene	48	—
2-Methylanthracene	50	—
9-Methylanthracene	54	—
1-Ethylphenanthrene	60	—
Fluoranthene	87	88
Pyrene	100	100
4-Methylpyrene	143	—
1-Methylpyrene	148	—
3-Methylpyrene	151	—
Benzo(*a*)fluorene	115	122
Benzo(*b*)fluorene	123	—
Benzo(*c*)fluorene	126	—
16,17-Dihydro-15H-cyclopenta(*a*)phenanthrene	121	—
17-Methyl-16,17-dihydro-15H-cyclopenta(*a*)phenanthrene	133	—
17-Methyl-15H-cyclopenta(*a*)phenanthrene	187	—
Triphenylene	264	—
Chrysene	263	245
Benz(*a*)anthracene	262	—
Naphthacene	284	267
2-Methylchrysene	376	—
3-Methylchrysene	374	—
4-Methylchrysene	348	318
6-Methylchrysene	384	—
1-Methylchrysene	390	356
7-Methylbenz(*a*)anthracene	409	372
Benzo(*e*)pyrene	—	668
Benzo(*a*)pyrene	—	729
Perylene	—	751
Benzo(*c*)tetraphene	—	1600
Picene	—	1610
Benzo(*g,h,i*)perylene	—	1760
Anthanthrene	—	1910

* Relative to pyrene = 100.
** 2.5%, 10 m and 2 m column.

1.0 mm capillary columns gave an HETP of 10 mm for chrysene at 200° with a sample load of 10^{-5} g. An outlet splitter was used, and fractions were collected for spectrophotometry.

Fraction collection

Fractions were collected in air-cooled glass tubes, the smaller of which (1 mm I.D.) were used for immediate melting-point determinations.

The collection efficiency was about 70 % for phenanthrene and the higher arenes at the 10^{-6} to $2 \cdot 10^{-5}$ g level but decreased to 50–60 % at 10^{-4} g because of fog formation. The recovery of the larger samples was improved (to 85–95 %) by electrostatic

TABLE IV

RELATIVE RETENTION TIMES OF ARENES ON SE-30 ON GLASS BEADS AT 200°

Compound	*Column*		
	(*a*)	(*b*)	(*c*)
Pyrene	1.00 (12 min)	1.00	1.00
Triphenylene	2.78	2.75	2.78
Benz(*a*)anthracene	2.77	3.25	2.77
Chrysene	2.81	3.5	2.81

(a) 2 m, 4 mm I.D., s/s (type 304) or 1 m, 3 mm I.D., Pyrex; 0.25% SE-30 on glass beads, 80–100 mesh.
(b) LIJINSKY *et al.*[27], 2.44 m (copper?); 0.25% SE-30 on glass beads.
(c) 2 m, 4 mm I.D., s/s (type 304), 10% SE-30 on Chromosorb W, acid washed, 80–100 mesh.

TABLE V

RELATIVE RETENTION TIMES OF ARENES ON CAPILLARY COLUMNS

Compound	*Column temperature* (°C)			
	260		235	
	(*a*)	(*b*)	(*c*)	(*d*)
Fluorene	*		0.24	
2-Methylfluorene	*		0.32	
Phenanthrene	0.44	0.45	0.42	
Anthracene			0.42	
3-Methylphenanthrene	0.56	0.56	0.55	
4,5-Methylenephenanthrene			0.60	
9-Methylanthracene			0.67	
Fluoranthene	0.87	0.87	0.87	
Pyrene	1.00**	1.00	1.00	1.00
4-Methylpyrene	1.38	1.38	1.43	
1-Methylpyrene			1.46	
2-Methylpyrene			1.34	
Benzo(*a*)fluorene	1.22	1.21		
Triphenylene			2.25	2.25
Chrysene	2.08	2.08	2.27	2.28
Benz(*a*)anthracene			2.24	2.24
4-Methylchrysene	2.80	2.79	3.13	3.15
Benzo(*e*)pyrene	4.60	4.58	5.43	5.45
Benzo(*a*)pyrene	4.72	4.72		

(a) and (c) 60 m, 0.5 mm I.D., s/s (type 347), SE-30 (*n*, $5 \cdot 10^4$, *n*-tetracosane; *n*, $2 \cdot 10^4$, chrysene);
(b) and (d) standard SE-30 analytical column.

* Retention times not accurately reproducible.
** 5 minutes.

precipitation, by scrubbing the gas by passing it through glass helices which were wet with ethanol at —20°, or by passing the gas through coarse (30 mesh) Celite.

Ultra-violet spectra have been determined as a routine procedure on injected samples of $2 \cdot 10^{-6}$ g for all the unsubstituted arenes of Table I from phenanthrene to coronene.

Detector response

The relative (flame ionization) detector response for the polynuclear arenes of Table I was found to vary by some ± 5 % depending on the sample size and the history of the column. In no case did it differ from unity by more than 10 %.

DISCUSSION

Evidently many of the polynuclear arenes can be resolved by gas chromatography under conditions attainable in a well-regulated chromatograph which is fitted with a sensitive (ionization) detector. The technique can be applied to samples of 10^{-6} g and less, a sensitivity which compares favourably with that of the liquid chromatographic methods.

For some purposes gas-chromatographic separation *alone* is adequate[31], but if (as is often the case) it is necessary, the concentrations of the unresolved components can be found either from the ultra-violet spectrum of the gas-chromatographic fraction (in a favourable case, such as that of the chrysene–benz(*a*)anthracene mixture) or by subsequent resolution (see below) of the fraction and determination of the ultra-violet spectra.

The following analytical scheme has proved satisfactory:

(1) Chromatography on alumina or silica gel, which serves to concentrate the polynuclear arenes.

(2) High-resolution gas chromatography on the phase SE-30, with the collection of fractions for ultra-violet spectrophotometry.

(3) (If necessary) chromatography on acetylated paper or acetylated cellulose of those fractions which are shown to be mixtures, and subsequent determination of the ultra-violet spectra of the resolved components.

This scheme has the advantage that the precision chromatography on the difficultly reproducible materials alumina, silica gel, and acetylated cellulose is replaced by high-efficiency gas chromatography, the reproducibility of which is good. Those compounds which are not resolved on SE-30 are readily separated on acetylated cellulose or acetylated paper (Table VI).

In qualitative analysis it is useful to know the relation between log (relative retention time) and carbon number (Fig. 1). It is apparent, however, that an alkyl carbon does not produce as great a log (retention time) increment as does a ring carbon. The retention values for the more highly alkylated arenes cannot therefore be derived from the data of Fig. 1.

The method outlined has given good quantitative results using the flame-ionization detector. Preliminary work has shown that the argon detector also is capable of giving an acceptable degree of precision. A disparity in detector response similar to that which LIJINSKY AND MASON[28] reported for the compounds pyrene and dibenz(*a*,*h*)anthracene (response values of about 1 and 0.01 respectively) has not been observed in this work with either the flame-ionization detector or the argon detector.

The retention values for the arenes have been found to be reproducible on three chromatographs provided that all are operated at the same temperature and not merely at the same *indicated* temperature. The effect of a difference in column temperature can, however, be minimized by the use of a retention reference compound with a retention volume close to that of the compound being studied.

TABLE VI

COMPARISON OF R_F VALUES OF ARENES ON ACETYLATED PAPER WITH RELATIVE RETENTION TIME VALUES ON SE-30

*Compound**	*Relative R_F value*		*Relative retention time*	
	(a)	(b)	(c)	(d)
Fluorene	4.00	4.30	0.33	0.28
Anthracene	3.55		0.50	0.457
Phenanthrene	3.90	4.78	0.50	0.453
Fluoranthrene	3.50	4.73	0.88	0.87
Pyrene	3.35	5.03	1.00	1.00
Benzo(*a*)fluorene	2.35	—	1.16	1.21
Benzo(*b*)fluorene	2.45	—	1.19	1.25
Benzo(*c*)fluorene	2.65	—	1.19	1.26
Benzo(*g,h,i*)fluoranthrene	3.20	—	1.68	1.84
Benzo(*c*)phenanthrene	2.75	—	**	**
Benz(*a*)anthracene	2.30	4.25	1.83	2.07
Triphenylene	—	5.37	1.82	2.07
Chrysene	1.50	2.21	1.83	2.08
Tetracene	—	—	1.95	2.21
Benzo(*b*)fluoranthene	1.43	—	**	4.01
Benzo(*j*)fluoranthene	1.70	—	**	**
Benzo(*k*)fluoranthene	1.95	—	**	4.04
Benzo(*e*)pyrene	2.70	—	3.64	4.58
Benzo(*a*)pyrene	1.00	1.00	3.74	4.72
Perylene	2.85	3.76	3.85	4.92
Indeno(1,2,3-*c,d*)pyrene	—	—	**	8.75
Dibenz(*a,h*)anthracene	2.50	—	6.32	8.96
Benzo(*c*)tetraphene	—	1.80	6.54	9.27
Picene	—	0.32	6.63	9.31
Dibenzo(*a,c*)phenanthrene	2.75	—	**	**
Benzo(*g,h,i*)perylene	3.35	—	7.11	9.88
Anthanthrene	1.20	1.75	7.53	10.50
Coronene	3.20	4.40	13.5	—
Dibenzo(*a,h*)pyrene	0.90	—	**	**
Dibenzo(*a,i*)pyrene	2.10	—	**	**
Dibenzo(*a,l*)pyrene	3.60	—	**	**

(a) Acetylated cellulose, HOFFMANN AND WYNDER[8].
(b) Acetylated cellulose, SPOTSWOOD[5].
(c) At 300° column temperature.
(d) At 260° column temperature.
* The compounds bracketed together are unresolved by chromatography on SE-30 at an efficiency of 10^4 theoretical plates.
** Relative retention time unknown.

The retention values differed only slightly whether determined on a kieselguhr-packed, a glass-bead-packed, or a capillary column (Tables I–V). There is, however, an unexplained discrepancy between certain of the values determined on the glass-bead column in this work and those reported by LIJINSKY *et al.*[27] (Table IV).

The use of capillary columns, and especially the 1 mm type which is useful for micro-preparative work, is a promising development and the overall time necessary for analysis of a polynuclear arene mixture can thereby be reduced considerably. A significant factor is the ease with which the capillary columns can be prepared. To utilize them to full advantage it will be necessary to study the problem of adsorption and means of overcoming it. The most likely method would seem to entail the use of a compact oxide-free metal rather than glass with its attendant disadvantages.

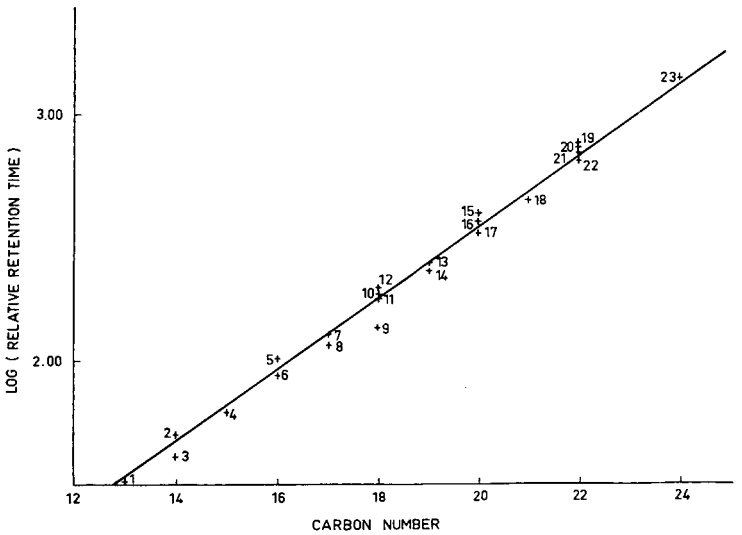

Fig. 1. Relation between relative retention time and carbon number. (1) fluorene; (2) phenanthrene; (3) 2-methylfluorene; (4) 3-methylphenanthrene; (5) pyrene; (6) fluoranthene; (7) 16,17-dihydro-15H-cyclopenta(*a*)phenanthrene; (8) benzo(*a*)fluorene; (9) 17-methyl-16,17-dihydro-15H-cyclopenta(*a*)phenanthrene; (10) chrysene; (11) benzo(*g,h,i*)fluoranthene; (12) naphthacene; (13) 2-methylchrysene; (14) 1-methylchrysene; (15) perylene; (16) benzo(*e*)pyrene; (17) benzo(*b*)fluoranthene; (18) 3-methylcholanthrene; (19) indeno(1,2,3-*c,d*)pyrene; (20) picene; (21) benzo(*g,h,i*)perylene; (22) anthanthrene; (23) coronene.

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SUMMARY

Gas chromatography has proved to be a convenient and rapid method for analysing mixtures of the polynuclear arenes. An analytical scheme is described for this analysis. Retention data are given for a number of these compounds on the phases SE-30, SE-52 and Q-F1. The use of packed and capillary columns has been investigated.

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IMPROVED EVALUATION OF POLYNUCLEAR HYDROCARBONS IN ATMOSPHERIC DUST BY GAS CHROMATOGRAPHY*

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In a previous paper[1] it has been reported that gas chromatography can be applied to the determination of polynuclear hydrocarbons in airborne particles.

The possibilities of gas chromatography for the evaluation of these compounds has been the object of further work and several aspects have been considered.

New liquid phases have been evaluated in order to obtain a sharper separation of certain compounds and programmed temperature chromatography has been used. It has also been shown that an electron capture detector may be used for the determination of polynuclear hydrocarbons and the relative response evaluated.

EXPERIMENTAL

A commercial gas chromatography apparatus (Fractovap C. Erba, Milano, model PAID/f) equipped with either a flame ionisation or an electron capture detector and a linear temperature programmer was used.

All measurements were carried out with glass capillary columns described previously[2]. The characteristics of the column are given in Table I.

TABLE I

COLUMN CHARACTERISTICS

Liquid phase	*Type*	*No. of theoretical plates*	*Length (m)*
(a) SE-30	Methylsilicone, Applied Sc. Lab.	20,000	30
(b) SE-52	Methylphenylsilicone, Applied Sc. Lab.	40,000	50
(c) XE-60	Cyanoethylmethylsilicone, Applied Sc. Lab.	20,000	35

The working conditions for these columns were: injector temperature 300°C; carrier gas: nitrogen at inlet pressure 0.3 atm. for (a) and (c) and 0.6 atm. for (b); splitter ratio 1:100, sample size 0.1–1.0 μg for each compound.

The retention volumes of some polynuclear hydrocarbons relative to chrysene obtained under isothermal conditions ($t = 200°$) are reported in Table II.

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** Research fellow of C.N.R.

TABLE II

RETENTION VOLUMES RELATIVE TO CHRYSENE AT 200°C, WITH GLASS CAPILLARY COLUMNS COATED WITH DIFFERENT LIQUID PHASES

Compound	*SE-30*	*SE-52*	*XE-60*
Anthracene	13	12	12
Fluoranthene	31	27	29
Pyrene	36	32	33
1,2-Benzfluorene	48	44	42
3-Methylpyrene	54	53	52
Benz(*m*,*n*,*o*)fluoranthene	82	79	81
1,2-Benzanthracene	98	97	98
Chrysene	100	100	100
Naphthacene	—	214	224
7,12-Dimethyl-1,2-benzanthracene	236	—	—
3,4-Benzfluoranthene	263	242	248
1,2-Benzpyrene	310	312	325
3,4-Benzpyrene	310	333	330
Perylene	321	363	336
20-Methylcholanthrene	400	—	—
1,3,5-Triphenylbenzene	415	455	315

The SE-30 column is not very specific and, as previously reported, some compounds are only slightly separated. Better results are obtained with an SE-52 column as a mixture of 1,2-benzpyrene, 3,4-benzpyrene and perylene give well defined peaks, and good resolution is also obtained for chrysene and 1,2-benzanthracene. These separations are of practical importance because of the toxic properties of these compounds.

The elution order and the elution time for the column coated with the more polar liquid XE-60 is approximately the same as for the SE-52 column. It cannot, however, be used at temperatures higher than 200°.

SE-52 has a low vapour pressure at high temperatures and does not bleed significantly up to 300°. It was found very useful for programmed temperature chromatograms. For a complete evaluation of polynuclear hydrocarbons, as is usually required in air pollution investigations, it is of interest also to determine compounds in a boiling point range higher than those reported in Table II; for this purpose programmed temperature chromatography was found very convenient. A good separation is realised for each compound and the analysis of a mixture of hydrocarbons, ranging in boiling point from naphthalene to coronene, is obtained as shown in Fig. 2.

RETENTION INDICES OF POLYNUCLEAR HYDROCARBONS

In order to make the data obtained in programmed temperature gas chromatography of general use and more suitable for the identification of polynuclear hydrocarbons found in complex mixtures, the retention indices, as described by KOVATS[3], were calculated.

In Fig. 1 the retention indices of polynuclear hydrocarbons are plotted against temperature, measured from a series of isothermal chromatograms run at different temperatures in the range 180–250°. These values have been calculated from the retention times of *n*-alkanes having from 16 to 28 carbon atoms.

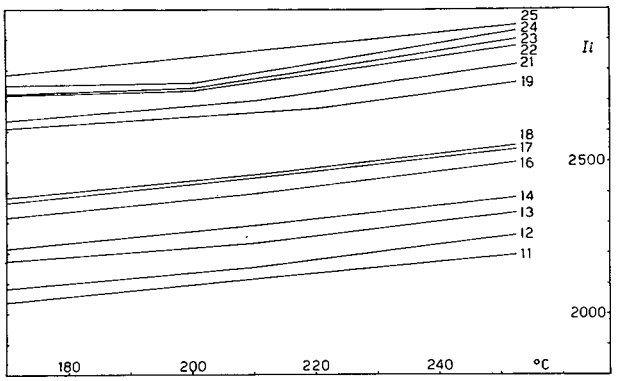

Fig. 1. Variation of retention index with the temperature. The numbers correspond to the compounds of Table III.

The retention indices are slightly affected by temperature variation. A change of less than 1 % is observed for an increase of 10°.

The retention indices for programmed temperature chromatograms (I_{pr}) have been computed from the retention temperatures (T_r) according to VAN DEN DOOL AND KRATS[4]. These values are collected in Table III and are in good agreement with the corresponding values of the isothermal indices (I_i) measured at a temperature 0.92 T_r, as is shown from the deviation, which is less that 12 units. The agreement between these figures indicates that both under isothermal conditions and at programmed temperatures, the identification of polynuclear hydrocarbons can be performed with a high degree of reliability.

APPLICATION OF THE ELECTRON CAPTURE DETECTOR

Many polynuclear hydrocarbons show a high affinity for free electrons and consequently, as reported by LOVELOCK[5,6], an electron capture detector can be employed for their chromatographic determination.

The interesting feature of a detector based on this principle is that its response to various hydrocarbons is quite different and thus its use can be very successful in the analysis of mixtures containing a large number of compounds, such as are usually observed in extracts of atmospheric dusts.

The weight response of some polynuclear hydrocarbons relative to benz-(*m*,*n*,*o*)-fluoranthene, measured with the electron capture detector, operating at 15 V D.C., is reported in Table IV.

These values differ widely and thus may be used to differentiate a certain component if the response ratio of the change in current in the flame ionisation and the electron capture detectors can be measured.

It should be pointed out, however, that the figures reported in Table IV are only indicative, because the response of an electron capture detector is greatly affected by geometrical characteristics and by the applied potential.

Fig. 3 shows the chromatogram of an atmospheric dust sample extracted according to the procedure described previously[1] and obtained with a flame ionisation and an electron capture detector.

TABLE III

RETENTION INDICES, ON SE-52, AT PROGRAMMED TEMPERATURES FROM 100° TO 300°C WITH PROGRAMMING 2.5°C/MIN.

n	*Compound*	T_r	I_{pr}	$I_i(0.92T_r)$	δ $I_i(0.92T_r)-I_{pr}$
1	Naphthalene	102	1173		
2	Azulene	105	1212		
3	Diphenyl	109	1262		
4	Acetylnaphthalene	113	1313		
5	Acenaphthalene	116	1350		
6	Fluorene	124	1460		
7	*trans*-Stilbene	137	1620		
8	Phenanthrene	143	1695		
9	Carbazole	154	1838		
10	Anthracene	160	1850		
11	Fluoranthene	174	1988	1977	−11
12	Pyrene	178	2040	2032	− 8
13	1,2-Benzfluorene	185	2125	2137	+12
14	3-Methylpyrene	189	2173	2176	+ 3
15	1-Methylpyrene	189	2173		
16	Benz-(*m*,*n*,*o*)-fluoranthene	199	2297	2297	0
17	1,2-Benzanthracene	204	2358	2356	− 2
18	Chrysene	207	2397	2394	− 3
19	Naphthacene	219	2543		
20	1,2-Benzfluoranthene	227	2648		
21	3,4-Benzfluoranthene	228	2655	2665	+10
22	1,2-Benzpyrene	234	2728	2728	0
23	3,4-Benzpyrene	235	2742	2738	− 4
24	Perylene	237	2766	2759	− 7
25	1,3,5-Triphenylbenzene	244	2853	2852	− 1
26	1,2,5,6-Dibenzanthracene	258	3008		
27	1,2,3,4-Dibenzanthracene	261	3048		
28	1,12-Benzperylene	278	3230		
29	1,2,3,4-Dibenzpyrene	281	3270		
30	Coronene	282	3283		

TABLE IV

WEIGHT RESPONSE FOR SOME POLYCYCLIC HYDROCARBONS WITH FLAME IONISATION (HF) AND ELECTRON CAPTURE (EC) RELATIVE TO BENZ-(*m*,*n*,*o*)-FLUORANTHENE

Compound	*HF*	*EC*	*EC/HF*
Anthracene	0.75	0.06	20.2
Fluoranthene	0.76	0.09	32.5
Pyrene	0.84	0.40	124.3
1,2-Benzfluorene	1.00	0.02	5.5
3-Methylpyrene	0.97	0.27	69.2
Benz-(*m*,*n*,*o*)-fluoranthene	1.00	1.00	250.0
1,2-Benzanthracene	0.90	0.87	267.2
Chrysene	0.85	0.00_5	1.5
3,4-Benzpyrene	1.53	2.15	343.5
1,2-Benzpyrene	0.66	0.75	310.0
3,4-Benzfluoranthene	1.01	0.67	180.2
Perylene	1.80	0.01	1.5

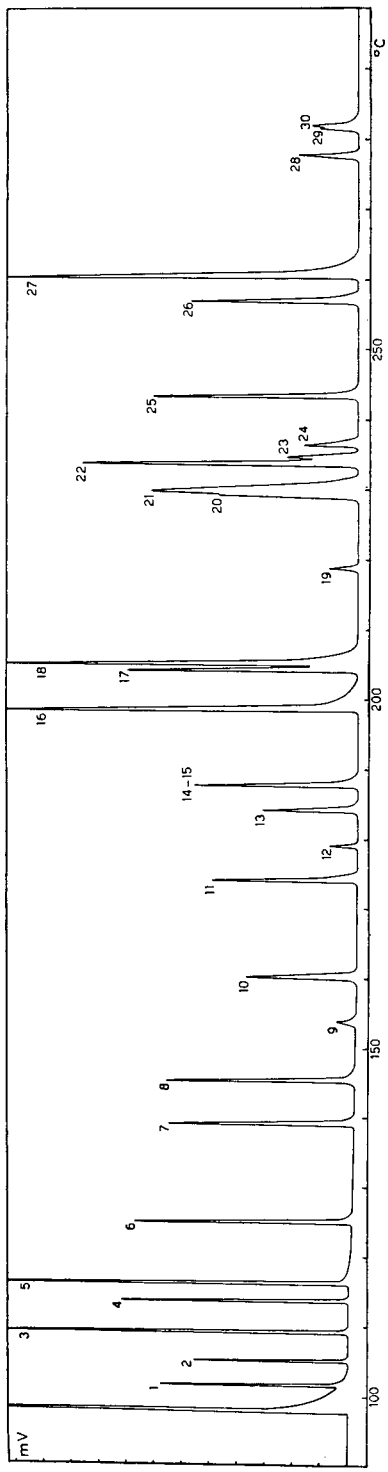

Fig. 2. Programmed temperature gas chromatogram of a polynuclear hydrocarbon mixture (100° – 300°C at 2.5°C/min). Glass capillary column coated with SE-52. The number of each peak corresponds to the compound reported in Table III.

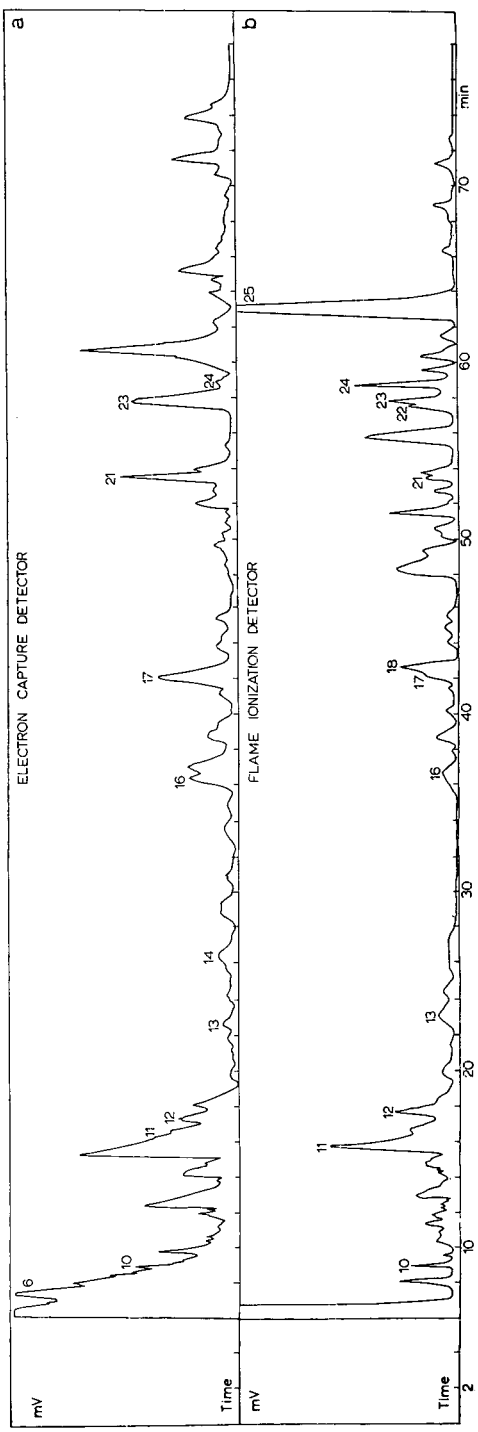

Fig. 3. Gas chromatogram of a representative sample of an atmospheric dust extract, obtained with EC (a) and HF (b) detectors. Programmed temperature 150–230°C at 2.5°C/min from 30–62 min on a SE-52 column. Numbers of the identified compounds as in Table III.

In the chromatogram of the atmospheric dust sample (Fig. 3) the response of the electron capture detector is very different from that of the flame and at the same time shows many other unknown compounds as large peaks that are not detected by the flame detector because of being present in too small an amount.

SUMMARY

Mixtures of polynuclear hydrocarbons can be analysed by means of high efficiency glass capillary columns. Several liquid phases have been tried, SE-52 being the most effective.

The retention index of these hydrocarbons has been measured isothermally and under programmed temperature conditions. An electron capture detector has been applied to the determination of such hydrocarbons and its relative response is compared with the results obtained with a hydrogen flame ionisation detector.

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TEMPERATURE DEPENDENCE OF THE HEIGHT EQUIVALENT TO A THEORETICAL PLATE

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INTRODUCTION

The height equivalent to a theoretical plate (HETP) represents, as is well known, the column efficiency in chromatographic separation. On the basis of the theory of LAPIDUS AND AMUNDSON[1], VAN DEEMTER *et al.*[2] derived a famous equation of the following form:

$$H = A + \frac{B}{u} + Cu \qquad (1)$$

In this equation H is the HETP and u the linear velocity of carrier gas.

KIESELBACH[3] and JONES[4] presented modified theories, and DE WET[5] found a hyperbolic relationship between HETP and temperature, for the elucidation of which he postulated that the diffusion constants in the gaseous and liquid phases are proportional to the absolute temperature and the partition coefficient K is inversely proportional thereto, resulting in the following expression:

$$H = A + B_3T + \frac{C_4}{T}$$

DUFFIELD[6] varied the linear velocity and found that the third term in the right-hand side of eqn. (1) increased.

In the present study it was deduced from the VAN DEEMTER theory[2] that the logarithm of HETP, and hence the logarithm of the plate number of a given column under certain conditions, varies linearly with the reciprocal of the absolute temperature.

THEORETICAL CONSIDERATION

LAPIDUS AND AMUNDSON[1], in 1952, presented a system of partial differential equations, in which diffusion, convection and the kinetics of dissolution into stationary phase and evaporation therefrom are considered mathematically. Their solution, however, was too complicated to be applied to the practical problems, and later VAN DEEMTER *et al.*[2] transformed it into a convenient form of the Gaussian distribution. Namely, it is shown that the outlet concentration is:

$$C_g = \frac{\beta t_0 c_0}{\sqrt{2\pi(\sigma_1^2 + \sigma_2^2)}} \exp\left\{ - \frac{\left(t - \frac{L}{\beta u}\right)^2}{\frac{2}{\beta^2}\left(\sigma_1^2 + \sigma_2^2\right)} \right\} \tag{2}$$

where t is the time, L the column length, and

$$\frac{1}{\beta} = 1 + \frac{F_l}{KF_g} \tag{3}$$

where F_g and F_l are the cross-sectional fractions of the gaseous and liquid phases, respectively, and K is the ratio of the concentration in the gaseous phase to that in the stationary liquid phase at equilibrium. It is considered that a pulse of duration t_0 and concentration c_0 is injected into the column inlet. The variances σ_1^2 and σ_2^2 are given by:

$$\sigma_1^2 = 2\frac{D_g L}{u^3} \tag{4}$$

and

$$\sigma_2^2 = 2\beta^2 \frac{F_l^2 L}{\alpha F_g K^2 u} \tag{5}$$

where α is the rate constant of dissolution from gaseous into liquid phase and D_g the diffusion constant in the gaseous phase.

It can easily be shown that the retention time t_R is given by

$$t_R = \frac{L}{\beta u} \tag{6}$$

The number of plates n is given by

$$n = \frac{L}{H} = \frac{t_R^2}{\sigma_1^2 + \sigma_2^2} \tag{7}$$

We will discuss the characteristics of eqn. (7) in two extreme cases.

At low temperatures

It is seen that in this case:

$$K \longrightarrow 0 \quad \text{and} \quad \frac{1}{\beta} \longrightarrow \frac{F_l}{KF_g} \tag{8}$$

so that the relation

$$\sigma_2^2 \gg \sigma_1^2$$

holds approximately. Hence, it follows that

$$n = \frac{\left(\frac{L}{\beta u}\right)^2}{\left(\frac{\sigma_2}{\beta}\right)^2} = \frac{L \cdot \alpha}{2 \cdot u \cdot F_g} \tag{9}$$

The rate constant of dissolution or adsorption α may be expressed in the form:

$$\alpha = \alpha_0 \cdot \exp\left(\frac{-\Delta H_1}{RT}\right) \tag{10}$$

where ΔH_1 is the activation heat of the process. It can therefore be derived that

$$\log n = \log \frac{L \cdot \alpha_0}{2 \cdot u \cdot F_g} - \frac{\Delta H_1}{2.303\ RT} \tag{11}$$

At high temperatures

In this case:

$$K \longrightarrow \infty \quad \text{and} \quad \beta \longrightarrow 1 \tag{12}$$

so that

$$\sigma_1^2 \gg \sigma_2^2 = 0.$$

Hence, it is seen that

$$n = \frac{t_R^2}{\sigma_1^2} = \frac{L \cdot u}{2 \cdot D} \tag{13}$$

On the other hand, one may write:

$$D = D_0 \cdot \exp\left(\frac{-\Delta H_2}{RT}\right) \tag{14}$$

Thus, it is found that

$$\log n = \log \frac{L \cdot u}{2 \cdot D_0} - \frac{\Delta H_2}{2.303\ RT} \tag{15}$$

or

$$\log H = \log \frac{2 \cdot D_0}{u} + \frac{\Delta H_2}{2.303\ RT} \tag{16}$$

EXPERIMENTAL

The benzene, toluene, isopropyl alcohol and ethyl alcohol used were all the reagent grade. The gas chromatograph was a Hitachi apparatus (Type KGL-24A). The column was 3 m long and 4 mm in diameter and the 10 % Apiezon grease L (40–60 mesh) served as the stationary phase. Helium was used as the carrier gas, and the pressure difference between the inlet and the outlet was maintained at 0.4 kg/sq. cm throughout the measurement. The amount of sample injected was always kept to 5 μl.

The variation of the linear velocity with temperature offers a complicated problem, but in this study it is assumed that the linear velocity of carrier gas remains approximately constant owing to the constant pressure difference. The change in the flow rate of gas volume expressed in mole/sec is measured and given in Fig. 1.

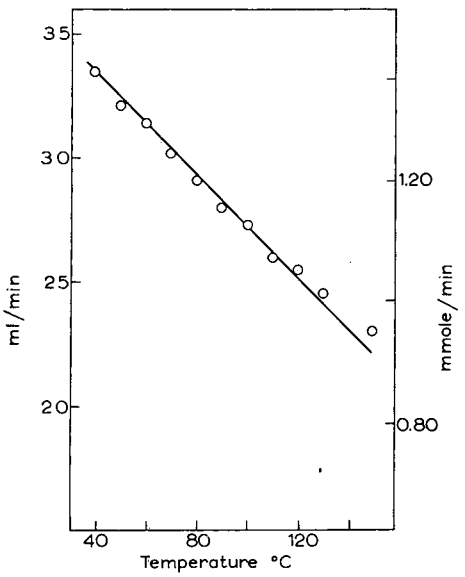


Fig. 1. Variation of the flow rate of helium carrier gas with temperature measured at 22° ± 1° and 1 atm at the column outlet. Under these conditions the logarithm of the flow rate varied only slightly.

RESULTS AND DISCUSSION

Some examples of the gas chromatograms recorded are shown in Fig. 2. It is found that with the increasing temperature the retention time becomes shorter and the peak first becomes sharper and then again broader.

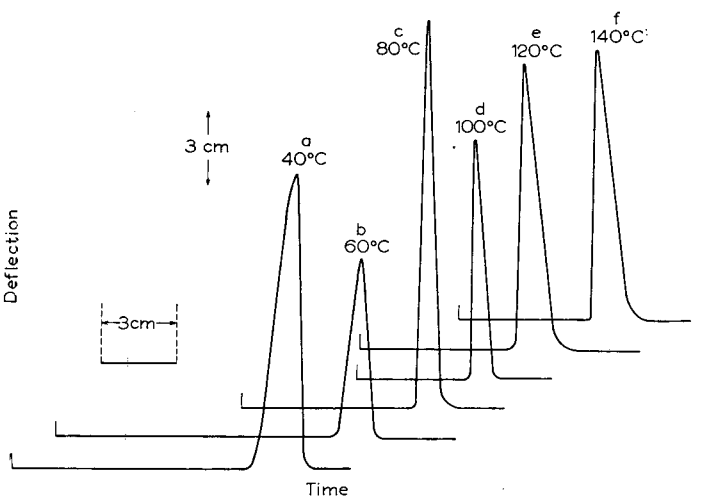
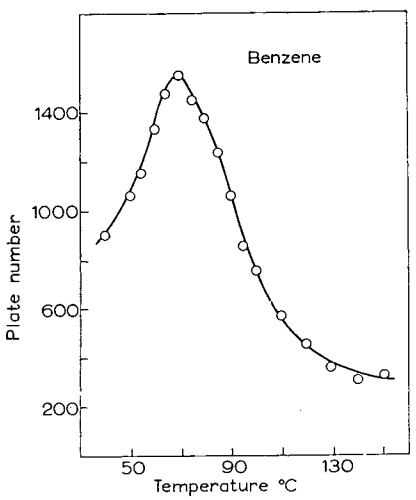
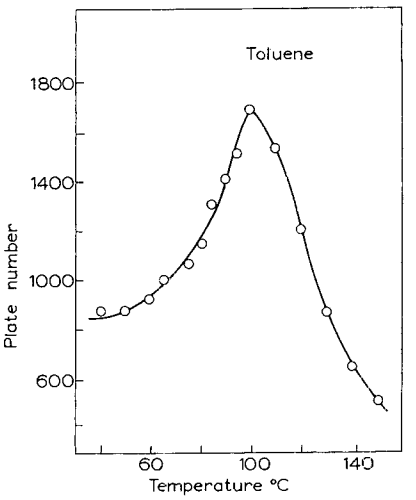


Fig. 2. Gas chromatograms of benzene at various temperatures. Recorder sensitivity (full-scale 23 cm) and chart speed are: (a) 8 mV, 0.5 cm min^{-1}; (b) 32 mV, 1.0 cm min^{-1}; (c) 32 mV, 1.0 cm min^{-1}; (d) 64 mV, 1.0 cm min^{-1}; (e) 64 mV, 2.0 cm min^{-1}; (f) 64 mV, 2.0 cm min^{-1}.





Figs. 3 and 4. Variation of plate number with temperature for benzene and toluene.

The number of plates was obtained by means of the equation:

$$n = 5.55 \left(\frac{t_R}{w_h}\right)^2 \tag{17}$$

where w_h is the half-width of the peak[7,8]. The variation of the number of plates with the temperature is shown in Table I and Figs. 3 and 4.

The logarithm of the plate number plotted against the reciprocal of the absolute temperature is shown in Fig. 5, from which one can easily find good linear

TABLE I

MEASUREMENT OF PLATE NUMBER AND RETENTION VOLUME

Column temperature (°C)	$1/T \times 10^3$	*Benzene*		*Toluene*	
		log n	*log* V_R	*log n*	*log* V_R
40	3.19	2.95	3.88	2.95	4.36
50	3.11	3.03	3.73	2.94	4.17
55	3.05	3.06	3.67	—	—
60	3.00	3.12	3.59	2.97	4.02
65	2.96	3.16	3.53	3.00	3.94
70	2.92	3.19	3.46	—	—
75	2.87	3.16	3.38	3.04	3.78
80	2.83	3.14	3.33	3.06	3.72
85	2.79	3.09	3.25	3.11	3.63
90	2.75	3.03	3.21	3.15	3.58
95	2.72	2.92	3.14	3.18	3.49
100	2.68	2.87	3.10	3.23	3.45
110	2.61	2.75	3.00	3.18	3.32
120	2.54	2.66	2.93	3.09	3.20
130	2.48	2.55	2.88	2.94	3.12
140	2.42	2.49	2.78	2.81	3.01
150	2.37	2.51	2.77	2.71	2.95

relationships indicating the essential validity of the present theoretical considerations.

The linear relationship between the log t_R and $1/T$ has been known for a long time, and is also given in Fig. 5. This would support the good control in the experimental work in the present measurement.

Compared with the theories proposed by DE WET[5] and DUFFIELD[6], the present study elucidates the temperature dependence of HETP, or of plate number in gas chromatography, more elegantly and reasonably and on the basis of sounder physicochemical considerations.

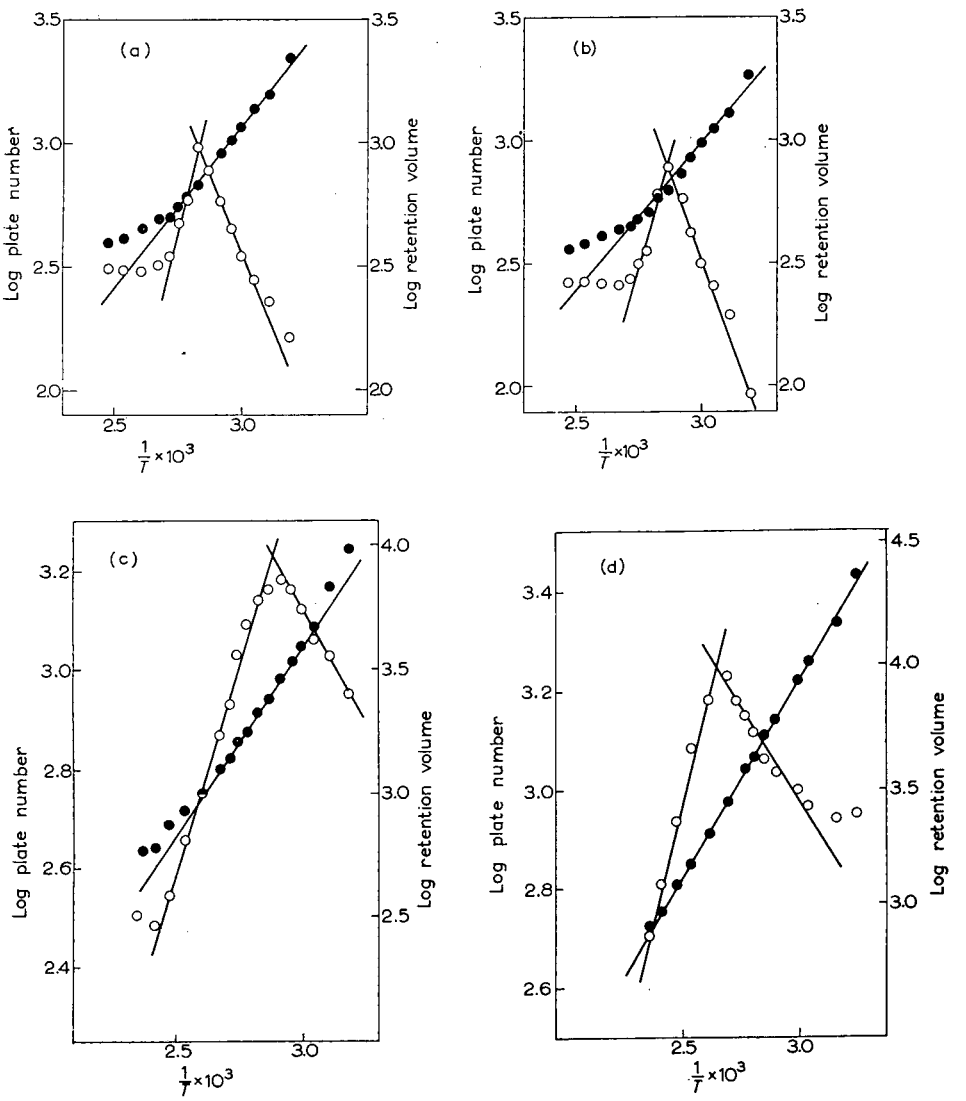


Fig. 5. Plots of log n *vs.* $1/T$ (shown by open circles). Log t_R is also plotted for comparison (shown by full circles). (a) Isopropyl alcohol; (b) ethyl alcohol; (c) benzene; (d) toluene.

SUMMARY

It was predicted theoretically that the plate number of a given column varies with temperature. From the VAN DEEMTER theory[2] it was shown that the logarithm of the plate number, or of HETP, plotted against the reciprocal of the absolute temperature gives two straight lines. This prediction was experimentally confirmed and it was found that each column shows the maximum plate number at a temperature near the boiling point of the sample.

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LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

X. ELECTRONIC AND STERIC EFFECTS IN HETERO-AROMATIC SOLUTES. SEPARATION OF THE DI-AZA-AROMATICS, QUINONES AND RELATED SOLUTES ON ALUMINA

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INTRODUCTION

Compound separability in linear elution adsorption chromatography (LEAC) over alumina has been examined previously for a variety of solute types[1–7]. Retention volume (ml/g) for elution by the solvent pentane $\underline{R}_p$ is related to adsorbent properties (activity function α and surface volume V_a) by means of:

$$\log \underline{R}_p = \log V_a + \alpha S^\circ \tag{1}$$

where S°, the dimensionless adsorption energy of the solvent from pentane onto calcined alumina, is a function of solute molecular structure only:

$$S^\circ = \sum_i^i Q^\circ_i + \sum_j^j q^\circ_j - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i \tag{1a}$$

The summation $\sum_i^i Q^\circ_i$ represents the first order contribution of all solute groups to solute adsorption energy, Q°_i being the contribution of each group i to S°. The summation $\sum_j^j q^\circ_j$ takes into account the effect of various intramolecular solute interactions j (or solute geometry factors) on solute adsorption energy; *e.g.* solute adsorption energy may be affected either by steric interactions between two adjacent solute groups, or by electronic interactions over longer distances. Finally, the term $f(Q^\circ_k) \sum^{i \neq j} Q^\circ_i$ corrects for adsorption energy changes which result from the localization of a strongly adsorbing solute group k on a particular adsorbent site. The effect of the eluent on solute retention volume has been summarized in a recent paper[3] in this series, and seems to be well understood, at least for alumina as adsorbent. The present paper will not be further concerned with the eluent role as such.

The most practical limitation on eqn. (1a) is occasioned by our limited tabulation of q°_j values for various solute geometries. In principle every new solute necessarily exhibits some unique geometrical relationship among its constituent atoms or groups, and we might expect as many q°_j values as solutes. In practice the problem is very much simpler, in that many solute geometry relationships have no notice-

able effect on adsorption energy or retention volume. An additional simplification results from our ability to classify some of the important (with respect to adsorption) solute geometry relationships and to relate them to fundamental molecular properties that have been studied in relation to other problems. At the present time there appear to be at least three general contributions of solute geometry *per se* to adsorption energy.

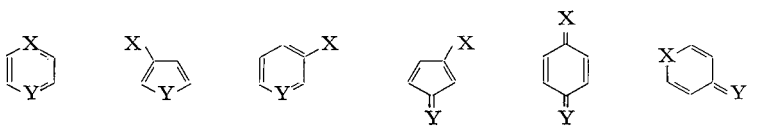
Electronic interaction between functional groups in the same molecule can have a profound effect on the adsorption energies of individual solute groups. In the *meta* or *para* substituted derivatives of pyridine[8], electron withdrawing substituents greatly reduce the adsorption energy of the pyridine nitrogen atom, and electron donating substituents increase its adsorption energy. This effect is reversed in the substituted indoles[6] and phenols[3], electron withdrawing groups increasing the adsorption energy of the –NH– and –OH groups in these two series of solutes. In all three of these cases, the effect of substituents on adsorption energy can be more easily understood by comparison with the related and well investigated phenomenon of electronic interaction in organic reactions.

Steric interaction between neighboring solute groups has long been known to have an important effect on solute adsorption energy. Steric effects can decrease solute adsorption energy by interference of one group with the adsorption of another. One example of this is in the adsorption of the phenyl-arenes[5]; steric interactions can either prevent the optimum planar adsorption of these solutes, or lower the net adsorption energy by creating an energy barrier to adoption of a planar configuration. In either case, solute adsorption energy is decreased. Similarly, bulky substituents adjacent to the nitrogen atom of the pyridine derivatives interfere with the adsorption of the nitrogen atom on strong alumina sites[6], again with a lowering of solute adsorption energy. In both of these experimental examples, the sterically induced loss in adsorption energy can be quantitatively correlated with the known interatomic distances and non-bonding interatomic potential functions. Steric effects can also affect solute adsorption energy by electronic interaction or bond formation between adjacent solute groups. In the adsorption of the hydroxyanthraquinones on silica[8], the 1-hydroxy derivatives are less strongly adsorbed than the 2-hydroxy compounds because the hydroxyl can hydrogen bond to one of the keto groups in the 1-derivatives, and this bond must be broken if the hydroxyl group is to interact with an adsorbent site. Finally, in the present and following papers, examples are provided of adjacent solute groups *increasing* solute adsorption energy by virtue of the fact that such groups may be able to simultaneously interact with a single, strong adsorbent site; a similar situation has already been noted in the adsorption of the unsaturated hydrocarbons on silica[9] and discussed theoretically.

Optimum positioning of the adsorbing groups within the solute molecule with respect to the topographical arrangement of strong adsorbent sites on the adsorbent surface (for optimum interaction of each adsorbing group with a strong adsorbent site) is also known to substantially affect solute adsorption energy. The previous example of adjacent solute groups interacting with a single adsorbent site is an obvious example of optimum adsorbing group distribution within the solute molecule. A more interesting example is provided by the preferential adsorption on silica of the solute dibenzyl[9], relative to the fused aromatic hydrocarbons of similar formula. In this case, the two phenyl rings can each interact in an optimum fashion with ad-

jacent strong adsorbent sites (see particularly, Fig. 10, ref. 7). Similarly, long aromatic hydrocarbons are preferentially adsorbed on alumina[4,5], relative to shorter, wider isomers, because the longer solutes have a better chance statistically of overlapping two strong adsorbent sites simultaneously. The following paper in this series provides some additional examples in the adsorption of the haloaromatics, and provides a more detailed analysis of this effect for adsorption on alumina.

Major electronic effects have not been observed in the adsorption of the polysubstituted benzenes on alumina[2], with the exception of the phenols[3]. Theoretically, electronic effects should be most important in solutes such as I–VI, and in such examples of these solute types as have been studied (substituted indoles, II; substituted pyridines, III) large electronic effects are noted. The further experimental



I II III IV V VI

study of this class of compounds seemed worthwhile from the standpoint of better understanding electronic effects in adsorption chromatography. The di-aza-aromatics, compounds with two pyridine-like nitrogens in a single molecule, were attractive in this connection. They include the type I solutes where strong electronic effects could be expected. Several of the di-aza-aromatics were commercially available, and we had already thoroughly studied the factors which determine the adsorption energy of the related pyridine derivatives. Furthermore, it was hoped that their study might resolve certain apparent anomalies[10] in the adsorption chromatographic separation of various *cis* and *trans* azobenzene derivatives. Thus, while *cis*-azobenzene is adsorbed more strongly than *trans*-azobenzene on alumina, the related *p*-bis-(phenylazo)-benzene stereoisomers show the relative adsorption strengths: *cis-trans* > *cis-cis* > *trans-trans*. Similarly, the adsorption strength on silica of the azopyridine stereoisomers reverses in going from the 2,2′-isomer (*trans* > *cis*) to the 3,3′-isomer (*cis* > *trans*).

The substituted fluorenones (type IV) and quinones (type V) also seemed worth studying, for similar reasons. The present communication describes the experimental and theoretical investigation of the separation on alumina of these various classes of compounds, as well as a general analysis of electronic effects in adsorption chromatography.

EXPERIMENTAL

The retention volume data cited in the present paper were obtained as previously[11]. The chromatographic activities of the adsorbents used are in terms of the revised activity scale[3]. Some difficulty was found in reproducing the $\underline{R}°$ values for 1,10-phenanthroline and 1-azaindole eluted by dioxan from 3.9% H_2O–Al_2O_3 (reported previously[3]). In some cases, these solutes did not appear to elute at all under these conditions, whereas previously[3] they were readily eluted. The reason for this anomaly has not yet been resolved.

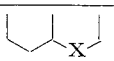
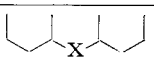
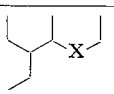
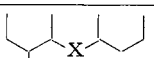
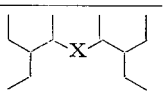
The eluent strength values ($\alpha\varepsilon^\circ$) required in extrapolating values of $\underline{R}^\circ$ to $\underline{R}_p$ were experimentally determined for the binary eluent mixtures as previously[11], and calculated for the pure eluents. The values used are tabulated in Tables III and IV.

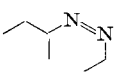
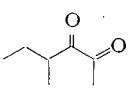
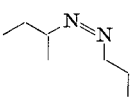
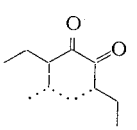
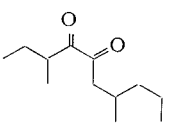
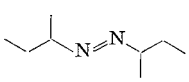
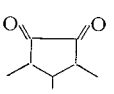
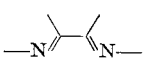
DISCUSSION

In following sections, the dependence of solute adsorption energy on steric and electronic interactions within certain classes of solute molecules is reviewed and experimentally extended. Almost without exception, a sufficiently complete understanding of these effects is possible so as to permit the quantitative calculation of solute S° values. The present discussion is intended as an intermediate summary of these results, for ready application to practical separation problems using adsorption chromatography on alumina.

TABLE I

SOLUTE PARAMETERS FOR AZA-AROMATICS, KETO-AROMATICS, FURANS, THIOPHENES AND PYRROLES

	Q°_i values versus solute geometry					
Group X						
–N=	4.8	4.0	3.8	2.3	0.7	(—0.1)
>C=O	(6.1)	4.7	3.8	3.4*	3.2	3.2*
–O–	1.1	0.3*	0.2	0.0*	0.0*	0.0*
–S–	0.7	0.6	0.6*	0.4*	0.1*	0.0*
–NH–	4.7	4.9	5.1	5.4*	5.6	5.6*

	Q°_i values for two hetero atoms of ortho grouping		
Group	*Exptl. Q°_i*	*Group*	*Exptl. Q°_i*
	5.5		5.3
	5.5		5.4
	6.5		5.8
	0.7		6.9
	7.4		

* Estimated values.

Table I summarizes $Q°_i$ values for five hetero-aromatic groups associated with the various derivatives of pyridine (–N=), cyclopentadienone (–C=O), furane (–O–), thiophene (–S–), and pyrrole (–NH–), as a function of the crowding of these groups in different solute geometries. All of these hetero group adsorption energies are seen to vary somewhat with increased crowding by adjacent solute groups: in the first four groups, increased crowding decreases adsorption energy, while in the pyrrole derivatives increased crowding increases adsorption energy. A previous discussion[6] has rationalized these differences in response of the hetero atom adsorption energy to crowding: normally, adsorption of the hetero atom X involves formation of a bond between X and the adsorbent, and increased crowding of X then reduces its adsorption energy; in the case of the pyrroles, the –NH– group functions as an acid which neutralizes the basic alumina surface, and increased crowding of the –NH– group weakens the strength of the H–N bond, which mildly increases pyrrole acidity and adsorption strength. These $Q°_i$ values of Table I should provide some insight into the variation of adsorption energy of other solute types with intramolecular crowding of a ring-incorporated hetero group. Substituent groups on an aromatic nucleus, as opposed to the ring-incorporated hetero groups of Table I, show less dependence of adsorption energy on intramolecular crowding[6]; a following paper will discuss these effects in detail. The $Q°_i$ values of Table I marked by * are actually estimates rather than experimentally derived values, based on plots of $Q°_i$ for one group *versus* $Q°_i$ values for the –N= group. Such plots show a rough correspondence between crowding and $Q°_i$ values for all of these hetero-aromatic groups. Using the $Q°_i$ values of Table I for these five hetero-aromatic groups, $S°$ values for the various benzo derivatives can be calculated from eqn. (1a) with the $q°_j$ term ignored; the only important solute geometry effect in these molecules is steric crowding of the hetero-aromatic group, and this is taken care of by the $Q°_i$ values of Table I.

The calculation of $S°$ values for solutes of the type I–VI must take into account not only the crowding of the groups X and Y, but their electronic interaction. The change in the adsorption energy of the group X as a result of interaction with the non-adjacent group Y may be defined as $q°_{x,y}$, and is given by the expression:

$$q°_{x,y} = \underline{A}\, Q°_x\, \sigma_y \qquad (2)$$

Here, σ_y is the HAMMETT σ value[12] for the substituent Y, a measure of its ability to relay an electronic charge to the position in the solute molecule occupied by the group X. $Q°_x$ is the adsorption energy of the group X, if it is the localized (strongest adsorbing) solute adsorbing group; if X is delocalized, $Q°_x$ refers to its $Q°_i$ value times the function $f(Q°_y)$, assuming that the group Y is localized. The parameter $\underline{A}$ refers to the response of the adsorption energy of X to changes in the charge of X. The calculation of $q°_{x,y}$ values is straightforward, but sufficiently tedious to be deferred to later sections. We will presently discuss only the more practical consequences of eqn. (2). First, and most important, is the question of whether the $q°_{x,y}$ values will be large; that is, does electronic interaction between X and Y significantly affect solute adsorption energy? This question is primarily answered by the values of $\underline{A}$ and $Q°_x$ involved for the solute type under consideration. Values of $\underline{A}$ appear to vary between $\pm$ 0.1 to 0.6, and are largest for solutes where the group X behaves as an acid or base upon adsorption (*e.g.* pyridines, phenols, etc.). For

solutes not adsorbing as an acid or base, a value of $\underline{A}$ equal —0.2 to —0.3 may generally be assumed. σ_y will generally be less than 1.0, so that for $q^\circ_{x,y}$ to be appreciable (0.1–0.2), Q°_x must be greater than 0.5–1.0, which means that electronic effects will normally be negligible for such solutes as the furan and thiophene derivatives (see Table I), at least as regards the adsorption energy of the hetero-aromatic groups –S– and –O–. Similarly, it is believed that electronic effects and $q^\circ_{x,y}$ values for most polysubstituted aromatic hydrocarbons will be small, and this has already been observed[2].

TABLE II

CALCULATED ADSORPTION ENERGIES S° FOR THE DI-AZA-AROMATICS CONTAINING ONE TO THREE FUSED RINGS

Di-aza-aromatic	*S° values for i,j di-aza-aromatic*				
	$i = 1$	$i = 2$	$i = 3$	$i = 4$	$i = 9$
Di-aza-benzenes					
$j =$ 2	8.0[a]				
3	6.3				
4	5.7[b]				
Di-aza-naphthalenes					
$j =$ 2	7.7[a,b]				
3	6.8	8.7[a,b]			
4	6.0				
5	7.0[b]				
6	8.2	8.5			
7	7.9	8.8			
8	(9.2)[c]				
Di-aza-anthracenes					
$j =$ 2	8.4[a]				
3	7.5	9.4[a]			
4	6.9				
5	8.3				
6	9.2	9.6			
7	9.1	9.7			
8	8.3				
9	(9.9)[c]	8.4			
10	7.8	8.8			6.5[b]
Di-aza-phenanthrenes					
$j =$ 2	8.4[a]				
3	7.5	9.4[a]			
4	6.3	6.9			
5	7.6[b]	8.2	8.1	10.3[a,b]	
6	9.0	9.7	9.5		
7	9.2	9.6			
8	8.4				
9	8.0	9.1	8.9	7.4	
10	(9.9)[c]	8.7	8.9	7.3	8.3[a,b]

[a] *ortho* derivative.
[b] Experimental value.
[c] Estimated Q°_i for two nitrogens intermediate between –N=N– and those at 1,10-phenanthroline.

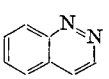
In the di-aza-aromatics and quinones, the combination of terms in eqn. (2) makes $q^\circ_{x,y}$ generally large, so that electronic effects tend to play an important role in determining the separability of various compounds in these two classes. $\underline{A}$ is negative for both the –N= and –C=O groups, and the σ_y values for these same two groups are positive, so that $q^\circ_{x,y}$ values for the quinones and di-aza-aromatics are always negative. As a result, introduction of a second –N= group into a mono-aza-aromatic or of a second keto group into a cyclopentadienone derivative to produce either a di-aza-aromatic or quinone, respectively, frequently results in a *net loss* in adsorption energy. Thus, pyrazine is adsorbed much less strongly than pyridine, while fluorenone and anthraquinone are adsorbed to about the same extent. The electronic interaction between X and Y decreases with the distance between the two groups, which means that the di-aza-aromatics or quinones with the hetero-aromatic groups in different rings (*e.g.* 1,5-di-aza-naphthalene, 2,7-naphthoquinone) are generally more strongly adsorbed than the corresponding derivatives with hetero-aromatic groups in the same ring (*e.g.* 1,4-di-aza-naphthalene, 1,4-naphthoquinone). Because of resonance, the *meta* di-aza-aromatics show smaller σ_y values in general, and are more strongly adsorbed. Thus, pyrimidine is predicted to adsorb more strongly than pyrazine.

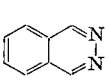
Whenever the solute groups X and Y are adjacent so as to permit simultaneous adsorption on a single adsorption site, S° is increased markedly. Thus, pyridazine is much more strongly adsorbed than pyrazine, and 1,2-naphthoquinone is more strongly adsorbed than 1,4-naphthoquinone. Table I summarizes some Q°_i values for the combined adsorption energy of two –N= or –C=O groups which form adjacent groupings of various types. The two nitrogens in 1,10-phenanthroline are seen to be adsorbed even more strongly than the bonded nitrogen pair of cinnoline or phthalazine, because of the more favorable arrangement of the 1,10-phenanthroline nitrogens for interaction with a single site.

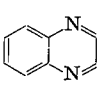
The variation of Q°_x with crowding in the di-aza-aromatics and quinones (as shown in Table I) is in general at least as important as the variation due to electronic and configuration (adjacency of groups) effects. Table II summarizes calculated or measured S° values for all of the one, two and three ring di-aza-aromatics for further illustration of the factors which determine adsorption energy in this series of compounds, as well as for use in estimating the separation of these and similar compound types.

THE DI-AZA-AROMATICS AS SOLUTES

The separation on alumina of the substituted pyridines and higher aromatic analogs (*e.g.* quinolines, acridines) has been studied in great detail[2,6], and an adequate understanding exists of the factors which determine the adsorption energy of the nitrogen atom in such cases. The di-aza-aromatics, compounds such as cinnoline (VII), phthalazine (VIII), quinoxaline (IX) and 1,5-naphthyridine (X), are closely related to the pyridine derivatives, and should therefore show similar adsorption characteristics. For this reason they were selected for initial study.


VII


VIII


IX

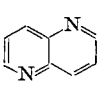

X

TABLE III

ADSORPTION OF THE DI-AZA-AROMATICS ON 3.8% H_2O–Al_2O_3

Solute	*log* $\underline{R}^\circ$	*log* $\underline{R}_p$	S°		Δ *exptl.*	σ[b]		$-\rho$		Δ *calc.*
			Exptl. eqn. (1)	*Calc.*[a] *eqn.* (2)		1[c]	2[d]	1[c]	2[d]	*eqn.* (3)
Pyrazine	0.57[e]	1.87	5.73	8.68	—2.95	0.76	0.76	2.68	1.47	—3.16
Phenazine	0.81[f], 0.29[g], —0.39[h]	2.36	6.50	8.79	—2.29	0.76	0.76	2.13	1.17	—2.51
5:6; 7:8-Dibenzoquinoxaline	0.61[f]	2.53	6.76	8.37	—1.61	0.57	0.57	1.29	1.00	—1.30
Dibenzo(*a*, *c*)phenazine	0.43[f]	2.67	6.98	7.60	—0.62	0.55	0.55	0.39	0.39	—0.43
1:2; 3:4; 6:7-Tribenzophenazine	0.63[f]	3.19	7.79							
1,5-Naphthyridine	1.03[e], 0.45[h]	2.70	7.03	8.40	—1.37	0.33[j]	0.33[j]	2.24	1.23	—1.15
4,4′-Dipyridyl	0.92[e], 0.51[h]	3.00	7.50	8.90[i]	—1.40	0.26	0.26	2.69	1.48	—1.08
m-Phenanthroline	1.03[e], 0.30[h]	3.08	7.62	8.15	—0.47	0.17[j]	0.17[j]	2.24	0.71	—0.50
Cinnoline	1.24[h], 0.01[k], —0.24[m]	3.13	7.70	9.20	—1.50	1.24	1.21	2.68	1.23	—4.80
Benzo(*c*)cinnoline	—0.05[k]	3.56	8.37	9.08	—0.75	1.12	1.12	2.24	1.23	—3.89
Phthalazine	1.15[k], 0.30[m]	3.74	8.66	9.64	—0.98	1.14	1.14	2.68	1.47	—4.96
1,10-Phenanthroline	2.03[h], 0.64[m]	4.77	10.27							
cis-Azobenzene	1.11[f], 0.43[g]	2.76	7.12							
trans-Azobenzene	1.03[n], —0.28[o]	1.03	4.42							

[a] Assumes Q°_i values of Table I.
[b] Calculated by the DEWAR-GRISDALE procedure for the corresponding hydrocarbon.
[c] For atom *n*.
[d] For atom *n*′.
[e] 50% v methylene chloride–pentane eluent ($\alpha\varepsilon^\circ$ = 0.207).
[f] 25% v methylene chloride–pentane eluent ($\alpha\varepsilon^\circ$ = 0.153).
[g] Benzene eluent ($\alpha\varepsilon^\circ$ = 0.209).
[h] Methylene chloride eluent ($\alpha\varepsilon^\circ$ = 0.275).
[i] Assumes contribution of —0.8 from non-planarity in solution.
[j] Times 0.7 as in Fig. 2.
[k] 50% v dioxan–pentane eluent ($\alpha\varepsilon^\circ$ = 0.361).
[m] Dioxan eluent ($\alpha\varepsilon^\circ$ = 0.411).
[n] Pentane eluent ($\alpha\varepsilon^\circ$ = 0.000).
[o] Carbon tetrachloride eluent ($\alpha\varepsilon^\circ$ = 0.118).

Table III summarizes retention volume data for several of the di-aza-aromatics on 3.8 % H_2O–Al_2O_3. As in previous studies of the mechanism of adsorption and the quantitative basis of retention volume in LEAC systems, it is useful to compare these experimental $S°$ data with values calculated by eqn. (1a), using the best adsorption parameters at our disposal. The difference $\Delta = (S°_{expt.} - S°_{calc.})$ can then be interpreted in terms of new effects of the type presently under study. The $Q°_i$ value of the nitrogen atom in the pyridine derivatives has been shown to vary with the intramolecular crowding by surrounding groups, and Table I summarizes the best values of $Q°_i$ for the nitrogen atom in various intramolecular configurations. Using these $Q°_i$ values from Table I and ignoring any $q°_j$ terms (which were initially unknown, and which lay at the center of the present investigation), $S°$ values were calculated for the solutes of Table III. For the solute 4,4'-bipyridyl, which is presumed to be non-planar in solution and to adsorb planar it was necessary to estimate the energy barrier to planarity. The previously measured[5] experimental value for biphenyl and related compounds (equal 0.8 $S°$ units for adsorption on 3.8 % H_2O–Al_2O_3) was assumed applicable.

As expected for the solutes of Table III, we find sizeable values of Δ in every case, reflecting strong electronic (and in some cases steric) interaction between the two nitrogen atoms of the di-aza-aromatics. The values of Δ are in every case negative; this might have been anticipated from the fact that electron withdrawing substituents on the pyridine ring lower the adsorption energy of the pyridine nitrogen, and the –N= group is itself electron withdrawing. The values of Δ vary widely among these various solutes, and it is important to establish the factors which determine these Δ values, and if possible set up a model for their quantitative prediction.

It has been shown[6] that the energy loss Δ in the substituted pyridines can be correlated by means of the HAMMETT equation[12]:

$$\Delta = \rho\sigma \tag{3}$$

where ρ is a parameter for a given organic reaction (the adsorption of a pyridine nitrogen atom on alumina in this case), determined by the response of the reaction to changes in electron density at the reaction site (the pyridine nitrogen atom). σ is a constant for each *meta* or *para* substituent, determined by the electron donating or withdrawing power of the substituent. For the adsorption of the substituted pyridines on alumina, ρ was measured[6] as -2.7 (converting from kcal to $S°$ units). We will now attempt to expand the correlation of the substituted pyridine adsorption energies to fit all of the related aza- and di-aza-aromatics.

The HAMMETT equation[12], eqn. (3), was first derived for *meta* and *para* benzenes and has since been empirically extended to other ring systems as well, although there have been relatively few correlations for non-benzene derivatives. DEWAR AND GRISDALE[13] have recently proposed a general formulation of the HAMMETT equation for application to non-benzene ring systems, and have shown it is possible to calculate σ values for the substituted naphthalenes and biphenyls which are in good agreement with experimental values. Briefly, if F and M are substituent parameters (values of these two parameters have been tabulated for a great number of aromatic substituents[13]), then σ in *any* ring system is given by:

$$\sigma = F/r_{ij} + M\, q_{ij}$$

where r_{ij} is simply the distance between the ring carbon bearing the substituent (j) and the ring carbon adjacent to the reaction center (i), and q_{ij} is a fundamental quantum mechanical property of the molecule (the formal charge created at position j by introducing a $-CH_2-$ group at position i). Both r_{ij} and q_{ij} turn out to be extremely simple and fast to calculate, so that with given values of F and M, the σ values of any substituent in many ring systems are readily obtainable.

Consider next the application of the DEWAR-GRISDALE treatment to the calculation of σ for the pyridine –N= group in various ring systems. Assume as a first approximation that the r_{ij} and q_{ij} values of the heteroaromatics are the same as those of the corresponding aromatic hydrocarbons (*e.g.* equal for naphthalene, quinoline and 1,5-naphthyridine). JAFFÉ[14] has summarized experimental values of σ for various positions of the pyridine and quinoline rings, relative to benzene. Considering only the 3- and 4-positions of these two molecules, best values of F and M for the –N= "substituent" can be derived: F equal 1.04 and M equal 1.67. Values of σ for the –N= groups in the various molecules of Table III can now be calculated in a straightforward fashion.

The next problem is the calculation of ρ for these and related solutes. Since the reaction center (*i.e.* a pyridine type –N= group) is the same in these various solutes, we might initially assume that the values of ρ are the same. We have seen, however, that Q°_i for these various groups is a function of solute structure, decreasing with increased intramolecular crowding. The contribution of a substituent group to the adsorption energy of a –N= group, and ρ, would also be expected to decrease with Q°_i of the –N= group. Similarly, when one –N= group in a di-aza-aromatic is localized on a strong site (as is generally the case[2]), the adsorption energy of the other –N= group is decreased to the effective value $f(Q^\circ_n)\ Q^\circ_{n'}$ where Q°_n is the value of Q°_i for the stronger adsorbing nitrogen n, $f(Q^\circ_n)$ is the value of the localization function for n, and $Q^\circ_{n'}$ is the value of Q°_i for the weaker adsorbing nitrogen n'. As a first approximation, then, we might expect ρ for localized –N= groups to be given by:

$$\rho = \underline{A}\ Q^\circ_n \tag{4a}$$

and ρ for non-localized (delocalized) –N= groups to be given by:

$$\rho = \underline{A}\ f(Q^\circ_n)\ Q^\circ_{n'} \tag{4b}$$

where $\underline{A}$ is a constant for the adsorption on alumina of any –N= group. From the value of ρ for the substituted pyridines (—2.7) and Q°_i for the pyridine nitrogen group (4.8), we calculate $\underline{A}$ equal —0.56.

Consider first the initial four solutes of Table III, which are all derivatives of pyrazine. Values of σ for each nitrogen in the pyrazine derivatives are listed, as calculated by the DEWAR-GRISDALE procedure for the corresponding hydrocarbon derivative, assuming the prior derived values of F (1.04) and M (1.67). Values of ρ from eqns. (4a and 4b) are also listed (assuming $\underline{A}$ equal —0.56). Finally, calculated values of Δ are shown, and we see that these agree with the experimental Δ values within an average deviation of about ± 0.2 S° units. This agreement is certainly within the experimental accuracy of the solute S° values, considering the necessity of extrapolating $\underline{R}^\circ$ values to get values of $\underline{R}_p$.

Calculation of an $S°$ value for the fifth solute of Table III, 1:2;3:4;6:7-tribenzophenazine, requires a value of $Q°_i$ for the more hindered nitrogen atom, which is unavailable. By trial and error calculation of $S°$ and Δ for this solute, we can, however, estimate the best value of $Q°_i$ for the more hindered nitrogen, equal —0.1. This appears to be a reasonable value of $Q°_i$ in that the normal bonding of the nitrogen to the adsorbent[6] is almost completely precluded, and some slight deactivation of the surrounding aromatic carbon atoms by this –N= group would be expected[2].

The next three solutes of Table III, beginning with 1,5-naphthyridine, each have the nitrogen atoms contained in different aromatic rings. Before extending the previous treatment to the calculation of Δ value for these solutes, it is well to consider some data on the reactivity of the substituted quinolines. Values of σ for substituents in the 6- and 7-positions of quinoline have been reported by BACIOCCHI, ILLUMINATI AND MARINO[15], while ELDERFIELD AND SIEGEL[16] have reported the σ values of the quinoline nucleus for reaction sites at the 6- and 7- (as well as other positions). These experimental values are for various organic reactions in solution. These various σ values for the effect of a substituent in one ring on a reactive center in the other of quinoline are plotted in Fig. 1 *versus* values calculated by the procedure of DEWAR AND GRISDALE as previously. A good correlation is noted, but the slope of the curve is 0.7 rather than the theoretical value of 1.0. This reflects some difference in the way resonance and/or field effects are transmitted across the quinoline ring, relative to a naphthalene ring. Doubtless this property extends to the pyridine ring systems as well, but our initial derivation of F and M values for the –N= group from the pyridine ring system itself has resulted in the cancellation of these differences. For our present purposes it suffices to know that the values of σ we calculate for the interaction of two nitrogens in different fused rings must be multiplied by 0.7 (as in Fig. 1). In the case of 4,4'-dipyridyl in Table III, it is uncertain which basis we should use to calculate values of σ, and we have arbitrarily assumed no correction factor (as

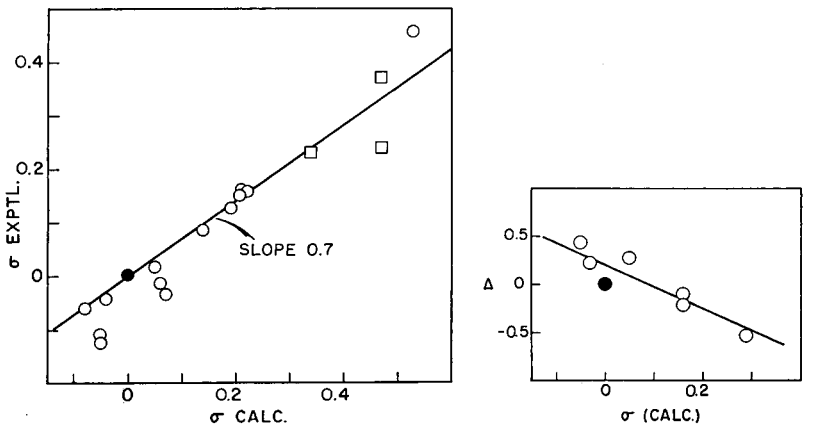


Fig. 1. Experimental σ values for 6- and 7-positions of quinoline *versus* values calculated from the DEWAR-GRISDALE model[13]. ○ = σ values for substituents in 6- and 7-positions of quinoline with reaction center at the 1-position[15]; □ = σ values for reaction center at 6- and 7-positions of quinoline[16].

Fig. 2. Correlation of Δ values in 6- and 7-substituted quinolines with σ.

in the pyrazine derivatives). Using values of σ and ρ calculated in this fashion and shown in Table III, the Δ values for 1,5-naphthyridine, 4,4'-dipyridyl and *m*-phenanthroline can be calculated. Again, we see agreement with experimental values which appears within the experimental error of the $S°$ values themselves (± 0.2 $S°$ units).

As a final example of the interpretation of electronic effects in the adsorption of the aza-aromatics by means of the present model, consider the Δ values of the 6- and 7-substituted quinolines which have been reported previously[5,6]. These data are plotted *versus* calculated σ values (corrected by 0.7) in Fig. 2, and the slope (value of ρ) of the best (least squares) line through these data is found to be -2.20. The calculated value of ρ (-0.56×4.0) equals -2.24. Again, excellent agreement is found between the experimental values of Δ for substituted aza-aromatics and values calculated from the DEWAR-GRISDALE model.

To emphasize the significance of the above correlations, it should be pointed out that the treatment followed was derived *entirely* and consistently from theory applicable to (and developed for) classical organic reactions. Thus, starting with $Q°_i$ values for the nitrogen atom in various configurations (Table 1) as measured from unsubstituted mono-aza-aromatics, it has been possible to quantitatively calculate $S°$ values for the first eight solutes of Table III, the six substituted quinolines of Fig. 2, and the 12 substituted pyridines reported previously[6]. Only one experimental parameter ($\underline{A}$) has been assumed. To the extent that we can similarly draw on the vast theoretical framework of physical-organic chemistry for other solute types where electronic effects affect adsorption energy, we can tremendously increase our ability to make quantitative calculations of solute adsorption energy (and separability). Because of the success of this treatment in the solute types so far examined, we can apply it to related solutes with considerable confidence. Table II summarizes calculated $S°$ values for all of the di-aza-aromatics containing 1 to 3 fused aromatic rings. As we will shortly see, the present treatment is inadequate to explain the Δ values of *ortho* di-aza-aromatics because of steric effects. The values of $S°$ for these solute types in Table II were estimated by other means.

It is seen in Table III that values of Δ calculated by eqn. (3) for the "*ortho*" di-aza-aromatics (cinnoline and the three following solutes) are significantly more negative than the experimental values (by an average of more than 3 $S°$ units). That is, these solutes are much more strongly adsorbed than predicted by simple electronic considerations. While there is some reason to believe that the DEWAR-GRISDALE treatment may be over-emphasizing the magnitude of electronic effects in the solutes where the nitrogens are bonded to one another, this in itself is an insufficient explanation of the discrepancy between experimental and calculated values. Thus, both TAFT[17] and CHARTON[18] estimate that the non-steric electronic effect in *ortho* and *para* substituted benzenes is of comparable magnitude (the DEWAR-GRISDALE model predicts that the electronic effect in the *ortho* position should be 50–100 % higher than in the *para* position), and if this were true, $S°$ for phthalazine would be predicted equal to about 6.4, whereas the experimental value is 2 $S°$ units greater. Similarly, 1,10-phenanthroline is not subjected to any uncertainty concerning the calculation of the electronic contribution to adsorption energy (the nitrogens are non-adjacent), and the calculated $S°$ value including electronic interaction ranges from 6 to 7, depending upon the crowding (and $Q°_i$ values) assumed for the nitrogen atoms. The actual value (10.27) is at least 3 $S°$ units higher. The only explanation that seems to

have merit in explaining the preferential adsorption of the *ortho* di-aza-aromatics is the assumption that both nitrogens in these solutes simultaneously interact with a single site, as pictured in Fig. 3 for the hypothetical adsorption of pyridazine. The well known tendency of 1,10-phenanthroline to chelate with metal salts is a similar phenomenon. As we shall see in the next section of this paper for the quinones, and in the immediately following paper of this series for the halo-aromatics, other solute types also show preferential adsorption of *ortho* type isomers when two adsorbing

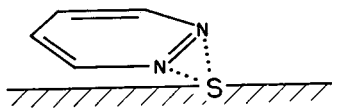


Fig. 3. Hypothetical utilization of same site (S) by both nitrogens in adsorption of pyridazine on alumina.

groups can share an adsorption site. Assuming that this explanation of the preferential adsorption of the *ortho* di-aza-aromatics is correct, it would seem appropriate to consider the two nitrogen atoms in such solutes as constituting a single functional group, from the standpoint of $S°$ calculations, with all $q°_j$ or Δ terms for this grouping combined into a single $Q°_i$ value for the two nitrogen atoms. Table I summarizes experimental $Q°_i$ values for the azo group in the various *ortho* di-aza-aromatics of Table III. Similarly, a value of $Q°_i$ for the two nitrogens (together) in 1,10-phenanthroline is given, equal 7.4. The very strong adsorption of this latter solute can be attributed to very favorable positioning of the two nitrogen atoms for simultaneous interaction with a single adsorbent site:

We have previously suggested[2] that the adsorption of 2,2'-dipyridyl gives a planar configuration essentially similar to that of 1,10-phenanthroline, rather than with nitrogen atoms lying on opposite sides of the adsorbed molecule. We can now calculate $S°$ values for each of the two conceivable (planar) configurations of adsorbed 2,2'-dipyridyl with some confidence and hence arrive at the true configuration. For the planar configuration, with nitrogens unopposed, there is no energy barrier to planarity, and we can calculate $S°$ equal 5.6 by the previous treatment and the data of Table I. For the alternate planar configuration where the nitrogens are equivalent to those in 1,10-phenanthroline, ADRIAN[19] estimates an energy barrier of 2.0 kcal, and we can calculate $S°$ (for adsorption on 3.8 % H_2O–Al_2O_3) equal 7.6, in good agreement with the experimental value of 7.5[2]. While the closeness of the calculated and experimental values is fortuitous, this check constitutes good evidence for a 1,10-phenanthroline type configuration in adsorbed 2,2'-dipyridyl.

We can briefly summarize the factors that determine the relative adsorption energy (on alumina) of the di-aza-aromatics. First, the intramolecular crowding of individual nitrogen atoms plays a role as in the case of the mono-aza-aromatics. The more crowded the nitrogens, the lower will be the adsorption energy. Second, the greater the distance between the two nitrogens, as when the nitrogens are in different rings, the greater will be the adsorption energy. The most important exception is when the two nitrogens are linked together in an azo group. Third, di-aza-aromatics where the nitrogens are conjugated with each other as in the 1- and 4-positions of pyrazine will tend to be adsorbed less strongly, relative to unconjugated di-aza-aromatics such as pyrimidine. Finally, whenever the two nitrogens are in a position

favorable to simultaneous bonding with a single adsorbent site, adsorption energy will be much increased.

SEPARATION OF THE *cis* AND *trans* FORMS OF THE AZOBENZENES AND RELATED COMPOUNDS ON ALUMINA AND OTHER ADSORBENTS

JACQUES AND KAGAN[10] have recently reviewed the separability on various adsorbents of the *cis* and *trans* isomers of the azobenzene derivatives and related compounds. COOK[20] was the first to note that *cis*-azobenzene is more strongly adsorbed on alumina than the *trans* isomer, and this applies to a large number of the mono- and di-substituted azobenzenes as well (*e.g.* *m*-methyl, *m*-nitro, *p*-chloro, *p*-bromo, *p*-nitro, etc.). On silica, FREUNDLICH AND ZELLER[21] found the same separation order (*cis* > *trans*), while on charcoal *trans*-azobenzene is the more strongly held isomer. CAMPBELL *et al.*[22] found that on silica the *cis* isomer of 3,3'-azopyridine is more strongly held, while the reverse order holds for the adsorption of *cis* and *trans* 2,2'-azopyridine. Finally, COOK[20] has noted that for adsorption of the *p*-bis-(phenylazo)-benzenes, the adsorption strengths are *cis-trans* > *cis-cis* > *trans-trans*. The basis for selectivity in the separation of the *cis* and *trans* azobenzenes has received only brief theoretical attention[21,23], and no satisfactory explanation has yet been offered for the variation of adsorption order (*cis versus trans*) between different compounds on the metal oxide adsorbents.

FREUNDLICH AND ZELLER[21] explained the reversal in separation order of the azobenzenes between alumina and charcoal in terms of the hydrophilic and hydrophobic character of these two adsorbents, and the preferential solubility of *cis*-azobenzene in water and methanol and of *trans*-azobenzene in petroleum ether. This is not to say, however, that solvent effects are controlling separation order in the azobenzenes; our previous analysis of the role of the eluent[3] clearly contradicts this possibility. KLEMM *et al.*[23] postulated the greater adsorption affinity of *cis*-azobenzene on alumina (in contrast to the preferential adsorption of the *trans* isomer in the stilbenes) as due to the greater ease of forming a bond between the adsorbent and the *n*-electrons of the nitrogens in *cis*-azobenzene. In view of our preceding analysis of the adsorption energies of the di-aza-aromatics, it is instructive to compare the experimental adsorption energies of the azobenzenes with values calculated according to Table I. The experimental $S°$ values for *cis* and *trans* azobenzene are given in Table III. Before calculating $S°$ values for these two compounds, the sterically induced nonplanarity of *cis*-azobenzene must be taken into account. Proceeding as previously[5], we calculate that *cis*-azobenzene is adsorbed with the nitrogens in the plane of the adsorbent surface, and the two phenyl groups at an angle of about 60° to the plane of the surface. The energy loss relative to planar adsorption of this compound is calculated at about —1.0 $S°$ units (3.8 % H_2O–Al_2O_3), considering delocalization of the two phenyl rings. *trans*-Azobenzene is believed planar in solution[19]. Using the $Q°_i$ value for the azo group in benzo(*c*)cinnoline (5.5), we then calculate a value of $S°$ for *cis*-azobenzene equal to 7.4, which is reasonably close to the experimental value of 7.1 (Table III). Similarly, for *trans*-azobenzene we calculate $Q°_i$ equal 0.84 for the two azo nitrogens*,

* Assumes $Q°_i$ for each nitrogen atom same as in 3:4-benzacridine (0.7), electronic deactivation of each nitrogen by the other as calculated by the DEWAR-GRISDALE model, and no possibility of simultaneous interaction of both nitrogens with one single site.

and $S°$ for *trans*-azobenzene equal 4.6, again in reasonable agreement with the experimental value (4.4). The separation of the azobenzene steroisomers on alumina is thus well explained by the factors which determine the relative adsorption of the pyridines and di-aza-aromatics; this is basically the same explanation as that offered originally by KLEMM *et al.*[23].

In the case of azobenzenes substituted by *meta* or *para* groups such as methyl or the halogens[20], these groups can have very little effect on the adsorption energy of either *cis* or *trans* azobenzene, inasmuch as their $Q°_i$ values are quite small, and the retention of preferential adsorption for the *cis* isomer is therefore to be expected. For the stronger adsorbing nitro group ($Q°_i$ equal 2.75) this situation becomes less obvious, although since $S°$ for *cis*-azobenzene exceeds that of *trans*-azobenzene by 2.7 units, it is difficult to see how substitution of one nitro group into azobenzene could lead to a reversal of separation order. The experimental observations of COOK[20] on the separation of the substituted azobenzenes on alumina are thus qualitatively in agreement with our theoretical predictions.

In the case of the *p*-bis-(phenylazo)-benzene derivatives, assumption of adsorption of all isomers in the plane of the adsorbent surface (but with twisting of *cis* phenyl groups as in *cis*-azobenzene) clearly leads to the predicted order: *cis-cis* > *cis-trans* > *trans-trans*. Experimentally, *cis-trans* is held most strongly, and some explanation for this anomaly must be sought. After consideration of several possibilities, involving unique localization phenomena, mutual deactivation of the various nitrogen atoms by electronic interaction, etc., we believe the following explanation is the most likely. KLEMM *et al.*[23] originally postulated, in interpreting the adsorption order of the pyridine derivatives, that these solutes tend to adsorb in a tilted or vertical configuration, with the nitrogen atom closest to the adsorbent surface for optimum interaction with a surface site. This hypothesis has been shown[6] inapplicable to the adsorption of the pyridine derivatives, which appear to adsorb in an essentially planar configuration. In the case of *cis*-azobenzene, however, with twisting of the attached phenyl rings 60° out of the plane of the adsorbent surface, there is a considerable driving force to tilted adsorption as proposed by KLEMM *et al.*[23]. Thus, in the absence of tilting, the nitrogen in *cis*-azobenzene would be required to lie about 1.5 Å more distant from the adsorbent surface than in the case of the nitrogen of a planar aromatic such as pyridine. This would be expected to reduce the adsorption energy of the azo group markedly. At the same time, tilting of the phenyl rings in *cis*-azobenzene would not markedly affect their adsorption energy, which by virtue of twisting and delocalization is already fairly small. If adsorbed *cis*-azobenzene is then tilted so as to maintain the azo group close to the alumina surface, *para* substituents on either of the two phenyl rings must be considerably elevated above the adsorbent surface by a kind of lever effect, with resultant reduction in the adsorption energy of such *para* substituents. In *cis-cis-p*-bis-(phenylazo)-benzene then, it is conceivable that the second (delocalized) *cis* azo linkage adds very little to the adsorption energy of the solute (relative to the adsorption energy of *cis*-azobenzene), and steric strain between the various phenyl groups would similarly prevent any sizeable increment to $S°$ of this solute from the third phenyl group. We would predict an $S°$ value for the *cis-cis* isomer about equal to or only slightly larger than that of *cis*-azobenzene (7.1). In the case of the *cis-trans* isomer, a similar situation would apply to the *trans* azo group, its elevation above the surface would reduce its ad-

sorption energy to zero, but in this case rotation about the delocalized azo group would permit almost complete return of the third phenyl group into the plane of the surface. This would result in a significant contribution to $S°$ of the solute, (1–1.5 units) and we would predict that the *cis-trans* isomer would definitely be more strongly adsorbed than the *cis-cis*. A straightforward calculation of $S°$ for the *trans-trans* isomer gives a value equal to 7.0, which is slightly less than that estimated for the *cis-cis* isomer. The present hypothesis is thus consistent with the experimental separation order found in the *p*-bis-(phenylazo)-benzene isomers, although the complexity of the configuration of adsorbed solute (and of the relevant $S°$ calculations) makes quantitative predictions of separation order for such solutes generally difficult, and probably impossible in many cases. As a general observation, however, the present hypothesis of tilted adsorption suggests that *reversal of the normal adsorption cis > trans, is always possible in the azobenzenes whenever a strongly adsorbing group is introduced into the para position of one or both phenyl rings* (*e.g.* *p*-amino-azobenzene, 4,4′-diaminoazobenzene, 4,4′-azopyridine).

Finally, in the case of the azopyridines adsorbed on silica[20], we can not offer direct calculations for this adsorbent but it is interesting to note that the same separation orders are expected on alumina. Thus, for the 2,2′-azopyridines, the normal reason for preferential adsorption of the *cis*-isomer no longer applies, because in adsorbed *trans*-2,2′-azopyridine all nitrogens are in the highly favorable 1,10-phenanthroline configuration, rather than in the highly unfavorable 3:4-benzacridine configuration of *trans*-azobenzene. *cis*-2,2′-Azopyridine would thus certainly be more strongly adsorbed than *trans* on alumina, as in fact it is found to be on silica. In the case of 3,3′-azopyridine, the normal separation order could be expected to apply, since the pyridine nitrogens can rotate into the approximate plane of the adsorbent surface in the adsorbed *cis* isomer.

In summary, the various experimental data on the separation order in the azobenzene and azopyridine derivatives seem theoretically reasonable, and some basis for qualitative prediction in this area seems possible. In certain cases, however, as in the *p*-bis-(phenylazo)-benzenes, accurate prediction of relative separation order may not always be feasible because of the complexity of the adsorbed solute configuration.

THE QUINONES AND FLUORENONES AS SOLUTES

Because the carbonyl group is both strongly adsorbing and electron withdrawing, we should expect to find many of the electronic effects observed in the adsorption of the di-aza-aromatics duplicated in the quinones. Table IV summarizes retention volume data for the adsorption on 3.8 % H_2O–Al_2O_3 of several quinones and of some monocarbonyl compounds of related interest. Starting with the first five solutes of Table IV, we can calculate $Q°_i$ values for the keto oxygen in each of these configurations: 4.7 in perinaphthenone, 3.8 in fluorenone (3.7 for the same keto configuration in 2:3-benzofluorenone), and 3.2 in 1:2-benzofluorenone. Using the fluorenone value of $Q°_i$ for the keto oxygen (3.8) we can calculate $S°$ for benzophenone, and find Δ equal —0.55. The weaker adsorption of benzophenone can be attributed to intramolecular steric hindrance to planar adsorption. Using the procedure of ADRIAN[19], we estimate that in solution each phenyl ring is twisted out of the plane of the carbonyl

TABLE IV

ADSORPTION OF THE QUINONES AND SOME RELATED MONO-CARBONYL COMPOUNDS ON 3.8% H_2O–Al_2O_3

Solute	*log* $\underline{R}^\circ$	*log* $\underline{R}_p$	S°	Δ		σ	$Q^\circ_o + f(Q^\circ_o)\, Q^\circ_o{}'$
				Exptl.	*Calc.*[a]		
Perinaphthenone	1.16[b], 0.33[c]	3.16	7.75				
Fluorenone	1.31[d], 0.33[b]	2.60	6.87				
1:2-Benzofluorenone	0.76[f]	2.81	7.20				
2:3-Benzofluorenone	1.49[e]	2.99	7.48				
Benzophenone	2.13[g], 0.96[e]	2.18	6.22	—0.55			
1,4-Quinone	1.13[e]	1.93	5.83			4.5	
1,4-Naphthoquinone	1.58[d], 1.11[f], 0.46[b]	2.50	6.72	—3.10	—3.1	3.5	7.3
9,10-Anthraquinone	1.24[e], 0.90[f], 0.32[b]	2.61	6.89	—2.22	—1.7	2.4	5.9
5,12-Naphthacenequinone	1.40[f]	3.45	8.20	—1.41	—1.7	2.4	5.9
7,12-Benzanthracenequinone	1.40[e], 1.09[f]	3.02	7.53	—1.93	—1.7	2.5	5.6
1:2; 3:4-Dibenzoanthracene quinone	1.65[e], 1.40[f]	3.58	8.40	—1.72	—1.8	2.8	5.2
Anthanthrone	1.61[b], 0.20[c]	4.40	9.68	—0.80	—0.7	1.0	5.9
1,2-Naphthoquinone	0.82[c]	3.24	7.87				
Acenaphthenequinone	1.75[c]	4.44	9.75				
9,10-Phenanthrenequinone	0.79[c]	3.75	8.67				
5,6-Chrysenequinone	0.91[c]	4.41	9.70				
2,4,7-Trinitrofluorenone	1.26[b]	5.01	10.64	—0.77	—1.3	3.1	3.8
Anthrone	1.05[f]	2.71	7.04	0.17	0.4	—0.8	3.8
2-Aminoanthraquinone		4.91[h]	10.48	1.33			

[a] Calculated for electronic effects only, as described in the text.
[b] 40% v methylene chloride–pentane eluent ($\alpha\varepsilon^\circ = 0.214$).
[c] Methylene chloride eluent ($\alpha\varepsilon^\circ = 0.269$).
[d] 10% v methylene chloride–pentane eluent ($\alpha\varepsilon^\circ = 0.110$).
[e] Carbon tetrachloride eluent ($\alpha\varepsilon^\circ = 0.115$).
20% v methylene chloride–pentane eluent ($\alpha\varepsilon^\circ = 0.158$).
[g] Pentane eluent ($\alpha\varepsilon^\circ = 0.00$).
[h] Data of ref. 11.

group by 35–40°. From this we estimate as previously[5] that Δ in the system of Table IV equals —0.5 to —0.6 $S°$ units, in excellent agreement with the observed value.

With the $Q°_i$ values for a keto group in the above configurations, we can calculate $S°$ values for the quinones which have similar keto configurations (all but 1,4-benzoquinone and the *ortho* quinones of Table IV). As in the case of the di-aza-aromatics, subtraction of our calculated $S°$ values from experimental values gives Δ values, and it remains to be seen if these experimental Δ values can be rationalized with the known electronic interactions in these molecules.

Unfortunately, no σ values (or experimental data from which they may be derived) have been reported for the ring-incorporated keto group (as in fluorenone), and we cannot therefore derive values of F and M as was possible in the aza-aromatics for the –N= group. An additional complication in applying the DEWAR-GRISDALE treatment is the presence of five-membered ring compounds (the fluorenones), inasmuch as these do not lend themselves to the same simple quantum mechanical formulation used previously for the aza-aromatics (because the hydrocarbon analogues are non-alternates). Consequently, only a crude application of theory to the solutes of Table IV is possible. As a first approximation, we might use the F and M values of the $-CO-CH_3$ group to approximate those of the keto >C=O group. We must recognize in adopting these values, however, that the keto group of the fluorenones and the quinones differs from that of the acetyl substituent in being attached to the aromatic ring system at two points, and the same is true of the keto group as a reaction center. The DEWAR-GRISDALE treatment considers the transmission of electronic effects in solutes such as the disubstituted benzenes to be between the ring carbons attached to the substituent and the reaction center. Using the same convention in treating the ring-incorporated keto group, we see that four rather than one transmission routes must be considered in the activation of one keto group by the other in the quinones: each carbon attached to the activating (substituent) keto activates each carbon attached to the "reaction center" keto group. Thus, in calculating the σ value of one keto group in 1,4-quinone for activation of the other, we might consider the quinone molecule to be constituted as in XI, with resonance possible between the bonded carbons, but not across the functional group Z (keto group). The field effect from atom 2 could be transmitted to atoms 3 *and* 5, however.

XI

Thus, σ for either keto group is then given as $F(1/r_{2,3} + 1/r_{2,5} + 1/r_{5,6} + 1/r_{3,6})$ plus $M(q_{2,3} + q_{5,6})$, where the $q_{i,j}$ values apply to the "aromatic" system ethylene. Using DEWAR AND GRISDALE's values of F and M (0.7 and 1.2), we calculate σ for either keto group in 1,4-quinone equal 4.5. Similarly, we calculate the other σ values shown in Table IV in an exactly analogous fashion. Next, the adsorption energy of the oxygen(s) in the various fluorenone and quinone derivatives of Table IV can be calculated, analogous to either $Q°_n$ or $Q°_{n'}$ for the aza-aromatics. The sum of these oxygen adsorption energies, $[Q°_o + f(Q°_o)\ Q°_{o'}]$, is shown for certain of the solutes in Table IV. Now, if our theoretical development is valid, by analogy with the aza-

aromatics, the experimental values of Δ in Table IV should be equal to the quantity σ-$[Q^\circ_o + f(Q^\circ_o)Q^\circ_{o'}]\underline{A}$. In Fig. 4, this relationship is tested by plotting experimental values of Δ *versus* $\sigma\,[Q^\circ_o + f(Q^\circ_o)Q^\circ_{o'}]$: a quite satisfactory correlation is noted, with the slope of the plot (value of $\underline{A}$) equal —0.12. The calculated values of Δ in Table IV (considering only electronic effects) show an average deviation from experimental values of $\pm$0.2 units, which is reasonable agreement when it is realized that the

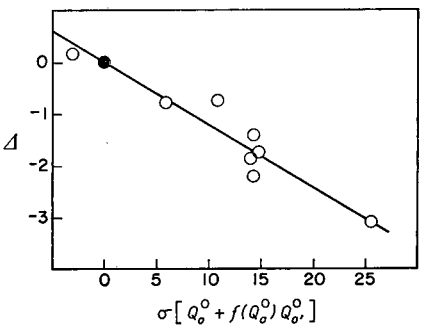


Fig. 4. Correlation of Δ values in substituted keto-aromatics and quinones with solute intramolecular electronic effects.

experimental values of S° (for different eluents) vary by $\pm$ 0.2 units average. To conclude, the extension of the DEWAR-GRISDALE treatment to the quinones gives a quantitative correlation of electronic effects on adsorption. The smaller value of $\underline{A}$ for the keto group adsorbing on alumina is partially due to the different basis used in calculating σ values. If we were to adopt the same convention in the aza-aromatics, this would lower $\underline{A}$ by a significant amount. The sensitivity of the keto group to electron withdrawal by substituent groups *is* less than that of the aza group –N=, however, as may be seen by comparing the Δ values in 6-nitroquinoline (—0.66) and 2,4,7-trinitrofluorenone (—0.77). That is, three nitro substituents in the second ring produce roughly the same adsorption energy loss in fluorenone as one nitro group in the second ring does in quinoline.

Extending the previous calculation to 1,4-quinone, we can calculate Q°_i for the keto group in the unstable cyclopentadieneone, equal 6.1. This value may be somewhat high because our adaptation of the DEWAR-GRISDALE treatment as above probably overestimates the σ value in 1,4-quinone (and also in 1,4-naphthoquinone). Table I summarizes these Q°_i values for the keto oxygen as a function of crowding, and there is seen to exist the same regular decrease in Q°_i with increasing crowding as in the analogous pyridine derivatives. Similarly, Q°_i values for the furan oxygen and thiophene sulfur groups are summarized in Table I, as derived from unreported $\underline{R}^\circ$ data on the benzo derivatives of these compounds. Again, we see the same trends in Q°_i with increased crowding of the ring hetero atom, although the magnitude of the effect in these latter solutes is less because the adsorption energy of the thiophene sulfur and furan oxygen is relatively small. In the case of the pyrrole derivatives, also reviewed in Table I, we see an opposite trend of Q°_i with crowding of the strong adsorbing group (-NH–), and this has previously been related[6] to the fact that the pyrroles adsorb as acids on alumina, whereas the pyridines and keto-aromatics have

been shown to adsorb with at least some electron transfer to the adsorbent. When an acid group becomes sufficiently crowded, however, as in 2,6-di-*tert.*-butylphenol[7], its group adsorption energy is also reduced. As a general rule, most organic solutes probably adsorb on alumina with electron transfer and show some reduction in group adsorption energy with increased crowding.

As in the case of the di-aza-aromatics, the *ortho* quinones are seen to be much more strongly adsorbed on alumina than the corresponding isomeric *para* quinones. Again, this may be attributed to the adsorption of both keto groups in the *ortho* quinones on a single adsorbent site. Values of $Q°_i$ for the combined two oxygens of the *ortho* quinones are shown in Table IV, as for the *ortho* di-aza-aromatics. These values are approximately constant (5.3–5.8) for the six-membered ring quinones, but acenaphthenequinone is considerably more strongly adsorbed ($Q°_i$ equal 6.9 for two oxygens). In the latter solute, where the keto groups are attached to a five-membered ring, it is theoretically expected that the mutual electronic deactivation of each keto group by the other will be reduced, relative to deactivation in the six-membered ring quinones, because of reduced resonance possibilities in a five-membered ring. The greater adsorption energy of acenaphthenequinone is hence at least qualitatively reasonable. The solute 2-aminoanthraquinone is a more complex example of electronic interaction between solute groups, and will be discussed in a following section.

Prior studies of the relative adsorption of the quinones on alumina and silica have been largely fragmentary, and no overall theory such as the present one has been previously attempted. HOYER (see ref. 8) has shown the importance of hydrogen bonding in determining the separation of certain anthraquinone derivatives on silica, and PETTERSSON[24] has recently reported thin-layer chromatographic data for separation of a number of benzoquinone derivatives on silica. Unfortunately, steric effects predominate in both these series of solutes, and comparison with our data (even overlooking the difference in adsorbent) is impossible. FUNAKUBO AND NAGAI[25] have studied the relative adsorption of anthraquinone, 2-methylanthraquinone and anthrone on both alumina and silica, and find adsorption increasing in this order for both adsorbents. They suggest that the greater adsorption of anthrone is due to the keto–enol equilibrium of this compound on the adsorbent, where the enol form would be expected to adsorb much more strongly. Our data suggest the same adsorption order found by the latter workers, since anthrone ($S°$ equal 7.04) is more strongly held than anthraquinone, ($S°$ equal 6.89) and the effect of a 2-methyl substituent in anthraquinone should slightly increase $S°$ (since the methyl group is electron donating). It is not, however, necessary to postulate a keto–enol equilibrium in anthrone; rather, in anthraquinone each keto group strongly suppresses the adsorption of the other, and in anthrone the carbonyl group is slightly activated by the electron donating methylene group. NAGAI AND FUNAKUBO[26] have also studied the adsorption of 1,2- and 1,4-naphthoquinone on alumina from hexane, in the non-linear isotherm region, finding the 1,4-isomer more strongly adsorbed. This observation, which does not agree with the data of Table IV, may be the result of non-linear isotherm adsorption, or of some peculiarity in the alumina used by these workers.

To summarize, our correlations of adsorption energy in the keto-aromatics and quinones show precisely the same type of electronic and steric interaction found in the aza-aromatics. Quantitative calculation of electronic effects in the keto-aromatics and quinones seems equally feasible.

ELECTRONIC AND STERIC EFFECTS IN OTHER SOLUTE TYPES

Having examined the effect of intramolecular steric and electronic interactions on the adsorption energy of the aza- and keto-aromatics, it is of interest to consider the existence of similar effects in the adsorption of other solute types. The substituted benzenes and naphthalenes have been examined previously[2], and to a first approximation it was concluded that electronic interactions between substituent groups in these solutes are unimportant in determining adsorption energy. A detailed examination of the effect of electronic and steric interactions in the adsorption of these solutes on alumina is planned for the following paper in this series. We can, however, make a few preliminary comments in the light of the present investigation. First, electronic interaction between substituents on an aromatic ring will always be less than interactions between a substituent on a ring and a functional group forming part of that ring (*e.g.* substituted pyridines); this is a simple consequence of the DEWAR-GRISDALE model, or of any reasonable theory of electronic effects in organic molecules. Second, the response of solute adsorption energy to intramolecular electronic interactions will always be greatest in those solutes where adsorption results in maximum transfer of charge to or from the solute. This means that electronic effects should be greatest for the adsorption of solutes functioning as acids (*e.g.* phenols) or bases (*e.g.* anilines) toward the adsorbent. In confirmation of this, we have seen that the Δ values for the substituted quinolines are about three times as great as for the substituted fluorenones. We can calculate ρ for the substituted acetophenones, a class of solutes which are probably typical of most substituted benzenes with respect to adsorption mechanism (the acetophenones do not adsorb as acids or bases), and which should give one of the largest ρ values for the non-acid non-base disubstituted benzenes, because Q°_i for the aceto group is relatively large (3.7). Extending the DEWAR-GRISDALE treatment for the keto-aromatics we calculate ρ for the substituted acetophenones equal —0.5. By comparison with some typical acidic and basic solutes, the substituted pyridines have ρ equal —2.7 and the phenols[3] +2.0. This implies that the effect of intramolecular interactions on solute adsorption energy will in general be small for the polysubstituted aromatic hydrocarbons.

In view of our comments on the large ρ values for the substituted acids and bases, the previously observed[6] value of ρ for the substituted anilines (—0.3) is anomalously small. The anilines and pyridines appear to possess a similar adsorption mechanism and the adsorption energies of the -N= group in pyridine and of the $-NH_2$ group in aniline are identical within experimental error. Extension of the DEWAR-GRISDALE calculations to the anilines would therefore have predicted a ρ value of about 1.0. As an explanation of the aniline adsorption data[6], it has been proposed that the anilines function as basic solutes when there are no strongly electron withdrawing substituents, and as acidic solutes when there are strongly electron withdrawing substituents. If this were in fact true, a plot of Δ *versus* σ for the anilines should go through a minimum, Δ increasing with σ at large values of σ, and Δ increasing as σ becomes more negative for negative values of σ. This postulate is tested in Fig. 5, where the Δ values of the substituted anilines[6] are plotted *versus* σ. The Δ value for 2-aminoanthraquinone is also included, since it gives a very large σ value (by the calculation of DEWAR AND GRISDALE, as previously). The resulting curve in Fig. 5 confirms our expectation, a definite minimum in the Δ values occurring

at intermediate values of σ. We tentatively conclude that the previous explanation for the dependence of Δ on σ in the anilines is correct.

Is has also been proposed[6] that the acetanilide derivatives adsorb as acids, by analogy with the acidic adsorption of some of the anilines. The value of ρ for the substituted acetanilides (—0.4) does not confirm this postulate, suggesting rather a dependence of solute adsorption energy on electronic effects which correspond to the adsorption of a weak base (with little actual charge developed on the solute). Possibly,

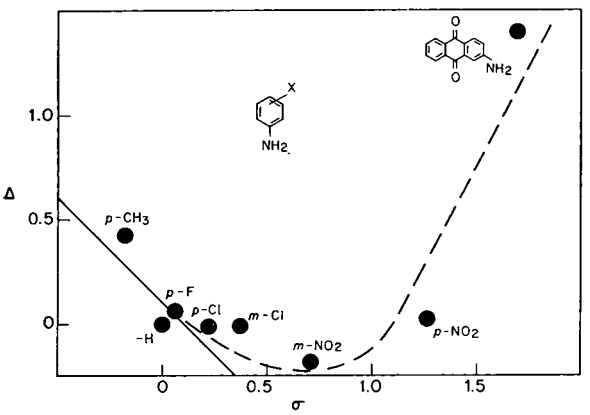


Fig. 5. Dependence of Δ in substituted aniline derivatives on σ.

because of steric hindrance to adsorption in the acetanilide amide group, the carbonyl is the principal contributor to the adsorption energy of the amide group; the observed value of ρ is about what would have been expected for this adsorption mechanism.

The monosubstituted hydrocarbons offer another example of electronic effects contributing to solute adsorption energy. In these cases, however, the effects are again rather small, and can be lumped into the $Q°_i$ value of the substituent group[2]. From the $q°_j$ values associated with the methyl- and ethyl-substituted benzenes[1], taking the $Q°_i$ value for a methyl group from aliphatic derivatives (—0.03), we can calculate the magnitude of the purely electronic effect in the monosubstituted benzenes as $\Delta = -0.6\,\bar{\sigma}$, where $\bar{\sigma}$ is the average of *meta* and *para* σ values for the substituent group in question. The corresponding value of $\underline{A}$ is —0.32; this value is roughly comparable to that (— 0.12) for the keto-aromatics, when it is realized that the actual σ values for the 1-, 2- and 6-positions in a monosubstituted benzene are probably underestimated by the function $\bar{\sigma}$. The adsorption of both the benzene nucleus and keto group on alumina are believed to occur with weak transfer of electronic charge from the solute to the adsorbent.

Some final examples of the electronic and steric interaction of adsorbing solute groups are summarized in Table V. Whereas the usual adsorption order in the di-aza-aromatics is 1,2 > 1,3 > 1,4, we see a reversal of this adsorption sequence for the aminopyridines. This may be caused by adsorption of the amino group as an acid when activated by strongly electron withdrawing substituents (such as the ring –N= group), and the activation of the ring –N= group by electron withdrawing substituents. Thus, in 4-aminopyridine the resonance structure XII is strongly

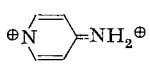
TABLE V

ADSORPTION ENERGIES ON ALUMINA OF SOME OTHER DIFUNCTIONAL SOLUTES; ELECTRONIC AND STERIC EFFECTS

Solute	S°
2-Aminopyridine	8.0[a,b]
3-Aminopyridine	8.2[a,c]
4-Aminopyridine	>9.3[a]
7-Azaindole	8.6[d]
1-Azacarbazole	9.5[a]
1,2-Diaminobenzene	9.2[e]
1,3-Diaminobenzene	8.8[e]
1,4-Diaminobenzene	8.8[e]
Dimethyl phthalate	8.2[e]
Dimethyl isophthalate	7.7[e]
Dimethyl terephthalate	7.6[e]
1,2-Nitroanisole	6.4[e]
1,3-Nitroanisole	5.6[e]
1,4-Nitroanisole	6.1[e]
1,2-Nitroaniline	7.4[e]
1,3-Nitroaniline	7.7[e]
1,4-Nitroaniline	7.9[e]

[a] Value from present study, dioxan elution from 3.9% H_2O–Al_2O_3.
[b] Previous values 8.3 (ref. 11) and 7.6 (ref. 3).
[c] Previous value 7.7 (ref. 6).
[d] Average of previous values 8.5 (ref. 11) and 8.7 (ref. 3).
[e] Data of ref. 2.

stabilized by adsorption of the –N= group on an acidic adsorbent site and the $-NH_2$



XII

group on a basic site, and as a consequence, the adsorption energy of this solute is considerably greater than calculated in the absence of electronic effects (calculated S° equal 9.1). While similar resonance structures may be drawn for 2-aminopyridine, this precludes adsorption of both nitrogen groups on the same site as postulated for pyridazine. The advantage of an *ortho* grouping of the two nitrogen atoms as in pyridazine is thus completely lost in 2-aminopyridine. Furthermore, the 2-amino group now partially interferes with the adsorption of the –N= group, as is the case in 2-methylpyridine[1,6]. Finally, there is a statistically greater possibility of the simultaneous adsorption of the amino group on an acidic site and of the –N= group on a basic site in the case of 4-aminopyridine than of 2-aminopyridine. The preferential adsorption of the 4-isomer relative to 2-aminopyridine is thus not surprising. The intermediate adsorption energy of 3-aminopyridine can be attributed to the absence of both resonance activation of the adsorbing groups as in XII and steric hindrance to adsorption of the –N= group as in 2-aminopyridine.

The adsorption energies of 7-azaindole, 1-azacarbazole and 2-aminopyridine show almost identical adsorption energies for the –N=C–NH– group (6.7, 7.0 and 6.8, respectively). The above explanation of the adsorption of the aminopyridines would have led to the prediction that the adsorption energy of the –N=C–NH– group in the above two indole derivatives would be greater than in 2-aminopyridine, since the additional vinyl substituent on the –NH– group in the indole derivatives should make this group even more acidic. Possibly involved here is a transition between adsorption of the –NH– group as a base in 2-aminopyridine (because of the preferential location of a strong site for adsorption in this fashion) and adsorption of the –NH– group in the two indole groups as an acid, with a relative independence of adsorption energy on the acid strength of the –NH– group, as in the substituted anilines. On this basis we would predict a considerably stronger adsorption of the non-*ortho* azaindole derivatives, as in the case of the aminopyridines.

Table V also summarizes previous data on the adsorption of those disubstituted benzenes where all three isomers were available. In the case of the diaminobenzenes, dimethyl esters of the benzene dicarboxylic acids, and nitroanisoles, the expected preferential adsorption of the *ortho* isomer is seen in every case. The situation is reversed for the nitroanilines, with the *ortho* isomer being least strongly adsorbed. If anything, electronic interactions through the ring would be expected to slightly increase the adsorption of *o*-nitroaniline. This seems to be a clear case of the *hydrogen bonding* between the amino and nitro groups decreasing the adsorption energy of the amino group, and possibly of the nitro group as well. In general, we would predict that *ortho* disubstituted benzenes in which intramolecular hydrogen bonding cannot occur, and where the adsorbing groups are capable of adsorbing on the same type site, will be preferentially adsorbed on alumina, relative to *meta* and *para* isomers.

GLOSSARY OF TERMS

$\underline{A}$ = A parameter which measures the relative change in the adsorption energy of a solute group i as a result of electronic activation by a substituent; equal ρ/Q°_i.

F = Parameter in the DEWAR-GRISDALE treatment which correlates the field effect of the substituent.

$f(Q^\circ_k)$ = Localization function for strong adsorbing group k.

M = Parameter in the DEWAR-GRISDALE treatment which correlates the resonance effect of the substituent.

Q°_i, Q°_x = Adsorption energy of solute groups i and x.

Q°_n = Adsorption energy of stronger adsorbing nitrogen atom in a di-aza-aromatic.

$Q^\circ_{n'}$ = Adsorption energy of weaker adsorbing nitrogen atom in a di-aza-aromatic.

Q°_o = Adsorption energy of stronger adsorbing oxygen group in a quinone.

$Q^\circ_{o'}$ = Adsorption energy of weaker adsorbing oxygen group in a quinone.

q°_j = Solute geometry factor.

$Q^\circ_{x,y}$ = q°_j value resulting from change in adsorption energy of group X by virtue of interaction with group Y.

q_{ij} = Electronic interaction factor for two atoms in a molecule (DEWAR-GRISDALE treatment).

$\underline{R}^\circ$ = Solute linear equivalent retention volume (ml/g).
$\underline{R}_p$ = Value of R° for pentane eluent.
r_{ij} = Separation of two atoms in a molecule (DEWAR-GRISDALE treatment).
S° = Solute adsorption energy; calcined alumina and pentane eluent.
V_a = Adsorbent surface volume (ml/g).
α = Adsorbent activity function.
Δ = Difference between calculated and experimental S° values, equal $S^\circ_{\text{exptl}} - S^\circ_{\text{calc}}$.
ε° = Eluent strength parameter.
σ, ρ = HAMMETT equation parameters.
$\bar{\sigma}$ = Average of σ values for *meta* and *para* substituents.
σ_y = σ value for group Y.

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SUMMARY

Hetero-aromatic solutes in which the hetero group is strongly adsorbing and forms part of the aromatic ring (*e.g.* pyridine, fluorenone) are markedly affected in their adsorption on alumina by intramolecular electronic and steric effects. Crowding of the hetero group by adjacent solute groups in these compounds results in strong reduction of the hetero group adsorption energy. The presence of electron withdrawing substituents in the derivatives of the fluorenones, quinones, di-aza-aromatics and pyridines also reduces the adsorption energy of the hetero group; because the nitrogen atom in the aza-aromatics and the carbonyl group in the quinones are strongly electron withdrawing, the di-aza-aromatics are generally no more strongly adsorbed than corresponding mono-aza-aromatics, while the quinones are about as strongly adsorbed as corresponding mono-carbonyl compounds. The presence of two strongly adsorbing solute groups in adjacent positions, however, as in the *ortho* quinones and pyridazine derivatives, strongly increases solute adsorption energy, because both solute groups can simultaneously bond with a single strong adsorbent site. Similar factors appear to determine the relative separability of the *cis* and *trans* azobenzene isomers. Using the theory of intramolecular electronic effects derived elsewhere from the study of organic reaction rates and equilibria, it is possible to quantitatively calculate the changes in solute adsorption energy which occur in the pyridines, di-aza-aromatics, fluorenones and quinones as a result of substituent electronic interactions. Electronic effects in other solute types are also examined theoretically, and concluded to be generally much smaller, with the exception of certain cases where the solute may function as either an acid or base. Less strongly adsorbing hetero-aromatic solutes (*e.g.* thiophenes, furans) show similar, but smaller responses to substituent steric and electronic interactions.

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THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

V. SEPARATION OF STEROL ACETATES BY THIN-LAYER CHROMATOGRAPHY IN REVERSED-PHASE SYSTEMS AND ON SILICA GEL G–SILVER NITRATE LAYERS*

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INTRODUCTION

The separation of steroids and sterols by means of thin-layer chromatography (TLC) has recently received much attention. The analysis of steroids was reviewed among others by WALDI[6] in the handbook of STAHL *et al.* Many recent investigations were published in the "Thin-layer chromatography issue" of the Journal of Chromatography[7]. The TLC of the lipophilic cholesterol esters has also been studied intensively by many authors, *e.g.* VAN DAM[8], HEŘMÁNEK *et al.*[9], MAHADEVAN AND LUNDBERG[10], WEICKER[11], JATZKEWITZ AND MEHL[12], KAUFMANN *et al.*[13], MICHALEC *et al.*[14], and ZÖLLNER AND WOLFRAM[15].

However, only a few of the investigations deal with the group of sterols and the related provitamins D, vitamins D, and triterpenoid alcohols.

The TLC of steroids, usually also comprising some sterol types, on silica gel G layers was studied by BARBIER *et al.*[16], VAN DAM *et al.*[17], WALDI[6], JANECKE AND MAAS-GOEBELS[18], TSCHESCHE AND SNATZKE[18], BENNETT AND HEFTMANN[20], NORMAN AND DE LUCA[21], and COPIUS-PEEREBOOM (see ref. 5, p. 97), while HEŘMÁNEK *et al.*[9,22] and ČERNY *et al.*[23] have studied such separations on spread layers of aluminium oxide. In general, mono-unsaturated sterols like cholesterol, provitamins D (*e.g.* ergosterol), and vitamins D are separable, but closely related sterols like cholesterol, stigmasterol, and β-sitosterol were not resolved.

The double bond isomers cholesterol and Δ^7-cholestenol were separated by BENNETT AND HEFTMANN[20] in the solvent cyclohexane–ethyl acetate–water (600:400:1).

AVIGAN *et al.*[24] also studied the application of TLC systems to the separation of related sterols. On normal silica gel G layers related sterols like cholesterol, β-sitosterol, and desmosterol were not resolved. A slightly better fractionation of cholesterol, Δ^7-cholestenol, 7-dehydrocholesterol, and lanosterol was achieved by chromatog-

* For parts I, II, III and IV of this series, see refs. 1, 2, 3, 4. A comprehensive survey of our work on the analysis of sterols is given in the thesis of one of us (C.-P.), *viz. Chromatographic Sterol Analysis*, Pudoc, Wageningen, 1963, ref. 5.

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raphy on 40 cm plates in benzene–ethyl acetate (20:1) for 24–36 h. AVIGAN *et al.* also reported the separation of lanosterol acetate and 24-dihydrolanosterol acetate on silica gel + 5% $CaSO_4 \cdot {}^1/_2\ H_2O$ layers using benzene–ethyl acetate (20:1) as mobile phase.

The behaviour of sterols on thin layers of aluminium oxide and kieselguhr G was studied by COPIUS-PEEREBOOM[3]. Using the solvent mixture cyclohexane–ethyl acetate (99.5:0.5) a mixture of ergosterol, cholesterol, and vitamin D_2 was fractionated. Cholesterol and Δ^7-cholestenol were resolved in the solvent cyclohexane–ethyl acetate (99.9:0.1).

In the above "normal" TLC systems the best separation was accomplished by BENNETT AND HEFTMANN[20] by analysing the trifluoroacetates of some sterols. The trifluoroacetates of cholesterol, stigmasterol, and desmosterol were separated with a cyclohexane–heptane (1:1) mixture.

The various systems for the separation of sterol acetates investigated by the authors are discussed below. After the authors' own investigations were finalized, BENNETT AND HEFTMANN[25] reported another procedure for separating β-sitosterol acetate, cholesterol acetate, and stigmasterol acetate, by means of continuous development with hexane–ether (97:3) on Anasil B plates for 120 min.

THE REVERSED-PHASE SYSTEM UNDECANE/ACETIC ACID–ACETONITRILE (1:3)

While studying the analysis of mixtures of animal and vegetable fats, we were mainly interested in the separation of closely related sterols like cholesterol, β-sitosterol, stigmasterol, and other phytosterols. In the above "normal" systems a separation between mono-unsaturated sterols like cholesterol and β-sitosterol could not be accomplished. However, reversed-phase thin-layer chromatography of the sterol acetates proved to be very suitable for this purpose.

After testing several reversed-phase systems, we finally decided that the system undecane (b.p. 190–220°)/acetic acid–acetonitrile (1:3) gives the best separations in the group of sterol acetates. The procedure of this reversed-phase system is given in the experimental part. In this system cholesterol and closely related phytosterols, *e.g.* campesterol, stigmasterol, and β-sitosterol, and in addition the pair cholesterol–dihydrocholesterol are clearly separated[3,4,5]. Analogous separations of the latter pair and of other positional isomers have since been reported by CARGILL[26], who has employed systems like undecane/methanol or/methanol–ether (49:1).

In such reversed-phase systems, both in paper chromatography and in thin-layer chromatography, the sterols or their acetates are arranged in several bands (see Fig. 1). The sterol acetates belonging to a certain band have nearly the same R_s (s = cholesterol) value and constitute a so-called critical pair.

The introduction of a double bond in the molecule causes nearly the same increase in R_s value as shortening the carbon chain by one methylene or methyl group. Average ΔR_M values were calculated from the R_M values of the compounds given in Table I, *viz.* $\Delta R_M{}^{C=C} = -0.08$; $\Delta R_M{}^{CH_2} = +0.06$.

The acetates of cholesterol (FC_{27}) from animal fats and of brassicasterol ($FC_{28}F^*$) from rapeseed oil, therefore, have nearly same R_s value and thus belong

* This formula means a C_{28} sterol skeleton with one double bond in the nucleus (FC_{28}) and another double bond in the C_{17}-side chain of the molecule ($C_{28}F$).

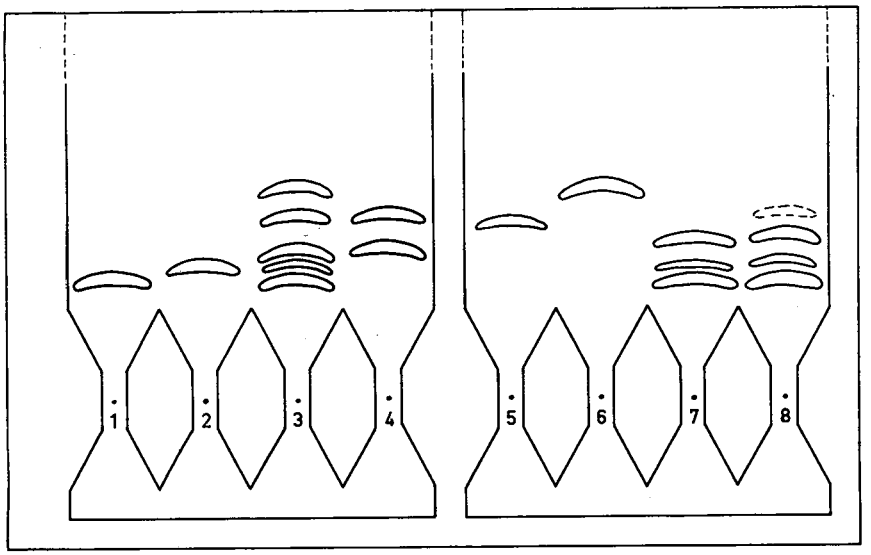

Fig. 1. Reversed-phase TLC of sterol acetates. Adsorbent: Kieselguhr G (Merck). System: undecane/acetic acid–acetonitrile (1:3). Time of run: 2 h. Detection: phosphomolybdic acid. Spot 1: 10 μg β-sitosterol acetates; spot 2: 10 μg stigmasterol acetate; spot 3: mixture of 1, 2, 4 and 6; spot 4: 20 μg of a mixture of cholesterol acetate–desmosterol acetate; spot 5: 10 μg ergosterol acetate; spot 6: 10 μg $\Delta^{9(11)}$-dehydroergosterol acetate; spot 7: 35 μg of phytosterol acetates from coconut fat; and spot 8: 35 μg of sterol acetates from *Asterina pectinifera*.

to the same critical pair. The equality of the migration rates of cholesterol, brassicasterol, and related $FC_{28}F$-sterols in this system hampers the suitability of our procedure, which was devised for the detection of animal fat in mixtures with vegetable fats. We were forced therefore to study the correlation between sterol structure and separability in more detail.

Naturally occurring complex sterol mixtures can be separated in this reversed-phase system and can be divided into six clearly separated bands, *viz.* critical pairs. The R_S values of some sterol acetates, classified according to these six critical pairs, are given in Table I.

The above arrangement of the sterol acetates into critical pairs is of course a rough classification. The position of the double bond in the nucleus and especially the occurrence of systems of double bonds will exert some influence upon the R_S value.

The R_S and also the R_M values are highly dependent on the experimental conditions of the applied reversed-phase system. A most unequivocal characterisation of the effect of introducing a double bond in a special position can be obtained by way of the so-called *carbon numbers* (*Nc*). This procedure has been applied by KAUFMANN AND MAKUS[27] to the separation of higher fatty acids and of triglycerides by reversed-phase systems of paper chromatography and of thin-layer chromatography. They introduced the so-called "papierchromatographische Wertzahl" (*pcW*), defined as the difference between the number of carbon atoms (n) and twice the number of double bonds (m) of the molecule, *viz.* $pcW = n - 2m$. However, such a procedure of calculating characteristic values, which are independent of the properties of the system, dates back to the so-called R_c values of DECKER[28].

TABLE I

R_s (s = CHOLESTEROL) VALUES OF STEROL ACETATES IN THE REVERSED-PHASE SYSTEM: UNDECANE/ACETIC ACID–ACETONITRILE (1:3)

Acetates of	*Shorthand designation*	*Band No.*	*Carbon number Nc*	*R_s value*
5α-Androstan-3β-ol	C_{19}		19.0	2.25
$\Delta^{9(11)}$-Dehydroergosterol	$3FC_{28}F$	6	22.7	1.45
Vitamin D_3	$3FC_{27}$	6	23.0	1.41
Dihydrovitamin D_2	$3FC_{28}$	5/6	23.6	1.34
Zymosterol	$FC_{27}F$	5	24.0	1.28
Vitamin D_2	$3FC_{28}F$	5	24.2	1.26
Desmosterol	$FC_{27}F$	5	24.35	1.24
3-Dehydrocholesterol	$2FC_{27}$	5	24.55	1.22
Ergosterol	$2FC_{28}F$	5	24.8	1.19
Epi-cholesterol	FC_{27}	5	24.8	1.19
7-Dehydrocholesterol	$2FC_{27}$	5	25.0	1.16
Ergosterol D ($\Delta^{7,9(11),22}$-ergostatrienol)	$2FC_{28}F$	5	25.4	1.11
Brassicasterol (7-dihydroergosterol)	$FC_{28}F$	4	25.8	1.07
5-Dihydroergosterol	$FC_{28}F$	4	25.8	1.07
22-Dihydroergosterol	$2FC_{28}$	4	25.4	1.07
Cholesterol	FC_{27}	4	≡ 26.0	≡ 1.00*
Lanosterol	$FC_{30}F$	4	26.0	1.00
Δ^3-Cholestenol	FC_{27}	4	26.0	1.00
Δ^7-Cholestenol	FC_{27}	4	26.5	0.99
Δ^7-Ergostenol	FC_{28}	3	27.0	0.92
Campesterol	FC_{28}	3	≡ 27.0	0.92
Stigmasterol	$FC_{29}F$	3	27.2	0.91
α-Spinasterol ($\Delta^{7,22}$-stigmastadienol)	$FC_{29}F$	3	27.2	0.91
Dihydrocholesterol	C_{27}	3	27.4	0.89
Agnosterol	$2FC_{30}F$	2/3	27.6	0.86
β-Sitosterol	FC_{29}	2	≡ 28.0	0.83
Dihydro-β-sitosterol (stigmastanol)	C_{29}	1	29.0	0.73

* The R_F value of cholesterol is about 0.28.

In the series of sterol acetates given in Table I the carbon number Nc is defined as $Nc = n - m$. Plotting the R_M values of the acetates of the mono-unsaturated sterols cholesterol ($Nc \equiv 26$), campesterol ($Nc \equiv 27$), and β-sitosterol ($Nc \equiv 28$) against their Nc values, a linear correlation is found. The R_M values of the saturated compounds: 5α-androstan-3β-ol acetate (C19) and dihydro-β-sitosterol acetate (C29) also obey this relation. In the R_M–Nc graph through these 5 points the straight line $R_M = 0.609\ (Nc - 19) - 0.09$ can be drawn. The R_M values of all other sterol acetates are interpolated and the corresponding Nc values are calculated graphically (see Table I). The Nc values can be considered as characteristic values, dependent only on the structure of the sterol molecule.

The Nc value belonging to an unknown component of a naturally occurring sterol mixture already gives some indication as to the possible structure of that component.

By means of reversed-phase TLC several hitherto unknown sterols have been detected in special sterol mixtures[1,5]. In many phytosterol mixtures isolated from edible vegetable oils, *e.g.* coconut fat, we could detect the presence of so-called *third-*

band phytosterols with an R_s value of 1.07 and *Nc* value of about 25.8 (see Fig. 1, spot 7). The structure of these special phytosterols, therefore, was supposed tentatively to be isomeric to that of methylcholestadienol, $FC_{28}F$.[5]

In chromatographic analysis the sterol mixtures from animal fats also show some peculiarities. Thus, in the sterols from hardened whale oil a band of $R_s = 0.88$ due to dihydrocholesterol was detected. In some crude and refined whale and fish oils two other sterol bands were observed with R_s values of 1.22 (*Nc* = 24.3), and 1.38 (*Nc* = 22.1), belonging to the "ergosterol critical pair" and to the "vitamin D_3 critical pair", respectively*.

The R_s values of Δ^7-sterols like Δ^7-cholestenol and 5-dihydroergosterol are identical to those of the corresponding Δ^5-sterols, *viz.* cholesterol and brassicasterol. Mixtures of homologous and related Δ^7-sterols are similarly separated in this system. The Δ^7-sterol mixture isolated from the starfish *Asterina pectinifera*** by TOYAMA AND TAKAGI[29] is separated into three bands with a very faint fourth band at $R_s = 1.22$ (see Fig. 1, spots 8)[5].

In the structural analysis of such complex sterol mixtures reversed-phase thin-layer chromatography may thus give circumstantial evidence concerning the structure of unknown sterol components. In many instances, however, various theoretically conceivable structures, all belonging to the same critical pair *e.g.* $FC_{27}F$ (with all possible isomers), $2FC_{27}$, $2FC_{28}F$, should be taken into consideration.

A separation of sterols according to principally different rules of separability, *e.g.* according to the "degree of unsaturation" of the components therefore appeared to be necessary. For this purpose several possible systems were investigated. Finally, two systems enabling a separation according to the "degree of unsaturation" were established, *viz.* the so-called "bromine-system" and silver nitrate TLC.

THE "BROMINE-SYSTEM"

KAUFMANN *et al.*[30] have described a procedure of separating fatty acids or triglycerides, belonging to the same critical pair but having a different number of double bonds, by the mere addition of 0.5 % of bromine to the mobile phase. In this way compounds with a different degree of unsaturation were separated. We have applied the principle of these so-called "bromine-systems" to the separation of the before-mentioned critical-pair partners cholesterol acetate–brassicasterol acetate and stigmasterol acetate–campesterol acetate[23].

FABRO[31] has described a paper chromatographic separation of the mono- and di-unsaturated sterols cholesterol and desmosterol by way of their bromo derivatives. A 10 % bromine solution was spotted directly on to the sterol sample at its starting point on the paper. MICHALEC[32] described a paper chromatographic separation of cholesterol and dihydrocholesterol, using a benzene–bromine (100:0.5) mixture. CARGILL[26] devised an analogous procedure of separating this sterol pair by TLC. After spotting the sterol sample at the starting point some drops of bromine were spotted on the same place. The plate was then developed with a benzene–ethyl

* More details concerning the analysis of such natural sterol mixtures will be published in a further communication of this series.

** We gratefully acknowledge the generous gift of the *Asterina pectinifera* sterol sample from Prof. TOYAMA.

acetate (2:1) mixture, by which means the spots of cholesterol and dihydrocholesterol were separated.

The R_s values of some sterol acetates in our "bromine-system", *viz.* undecane/acetic acid–acetonitrile (1:3) + 0.5 % of bromine are given in Table II. The identity of the bands of the dibromo derivatives which are made visible by spraying with antimony (III) chloride, was verified by a specific reaction with cadmium chloride[5].

The separability in this system is partly due to a normal reversed-phase separation of mono-unsaturated FC_{29}-, FC_{28}- and FC_{27}-sterol acetates, and partly to an enhanced migration of the more polar di-unsaturated sterols.

Because of both effects the migration rates of stigmasterol acetate and cholesterol acetate in this system are nearly equal. Likewise, the migration rate of brassicasterol acetate, present in rapeseed oil, is enhanced by a ΔR_s value of +0.06 to an R_s of 1.13, as compared with the "normal" reversed-phase system (see Fig. 2 and ref. 5, Fig. 34).

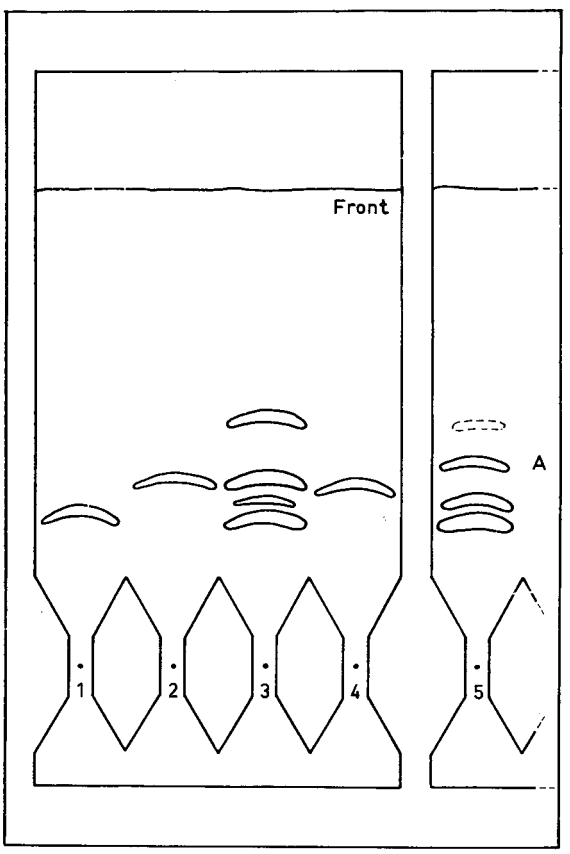

Fig. 2. TLC of sterol acetates in the "bromine-system". Adsorbent: Kieselguhr G (Merck). System: undecane/acetic acid–acetonitrile (1:3) + 0.5 % of bromine. Time of run: 2 h. Detection: heating, 50 % antimony (III) chloride. Spot 1: 40 μg β-sitosterol acetate; spot 2: 20 μg stigmasterol acetate; spot 3: 80 μg of phytosterol acetates from coconut fat; spot 4: 20 μg cholesterol acetate; and spot 5: 80 μg of phytosterol acetates from rapeseed oil (A = brassicasterol).

In the reversed-phase system in the previous section the R_s value of the third-band phytosterols from vegetable oils is equal to that of brassicasterol. In the bromine-system the third-band phytosterols, however, have a somewhat higher R_s value *viz.* 1.40 as compared to that of brassicasterol. In this way the three critical-pair partners cholesterol–brassicasterol–third-band phytosterols are clearly separated. In the bromine-systems compounds having a higher polarity, *e.g.* a higher degree of

TABLE II

R_s VALUES OF STEROL ACETATES IN THE SO-CALLED BROMINE-SYSTEM: UNDECANE/ACETIC ACID–ACETONITRILE (1:3) +0.5% OF BROMINE

Acetates	*Shorthand designation*	R_s *value*
Ergosterol	$2FC_{28}F$	front
7-Dehydrocholesterol	$2FC_{27}$	front
Third-band phytosterols from coconut fat	"$FC_{28}F$"?	1.40
Brassicasterol	$FC_{28}F$	1.13
Stigmasterol	$FC_{29}F$	1.06
Cholesterol	FC_{27}	≡ 1.00
Δ^7-Ergostenol	FC_{28}	front
Campesterol	FC_{28}	0.89
Dihydrocholesterol	C_{27}	0.85
Lanosterol	$FC_{30}F$	front
β-Sitosterol	FC_{29}	0.82

unsaturation, display a higher migration rate. The tentative $FC_{28}F$-structure for the third-band phytosterols should therefore have a higher polarity than that of brassicasterol with a double bond at 24 (28) or 25 (26) etc.

Sterols with a system of conjugated double bonds and sterols devoid of the normal $\Delta^{5(6)}$ double bond, *e.g.* Δ^7-sterols, lanosterol, and zymosterol, are completely decomposed in the bromine-system. Sterol structures with two or more conjugated double bonds like ergosterol, 7-dehydrocholesterol, vitamin D_3 etc. are not likely to be attributed to these third-band phytosterols.

By combining the R_s value of an unknown sterol in the normal reversed-phase and in this bromine-system respectively, ample circumstantial evidence concerning its structure can be obtained.

Δ^7-Sterol mixtures *e.g.* from *Asterina pectinifera* are similarly decomposed and only show blue spots near the solvent front, caused by decomposition products. Both Δ^7-sterols and "conjugated sterols" cannot be analysed in this system. The possibility of analysing sterols with "silver nitrate TLC", which is known to separate compounds mainly according to their degree of unsaturation without any decomposition effects, therefore had to be investigated.

SEPARATION OF STEROLS AND STEROL ACETATES ON SILVER NITRATE PLATES

In gas–liquid chromatography the admixture of silver salts to stationary phases like benzyl cyanide or glycols in order to enable a good resolution of paraffins and olefins is a well-known procedure.

The application of a silver nitrate coated adsorbent to the column chromatographic fractionation of lipids was described by DE VRIES[33,34]. The procedure was soon extended to thin-layer chromatography. MORRIS[35] and DE VRIES[36] succeeded in separating the methyl esters of oleic acid, linoleic acid, and linolenic acid, which differ in the number of double bonds. Furthermore, *cis–trans* isomers like methyl oleate and methyl elaidate and their corresponding epoxy and hydroxy esters were separated.

The fractionation of synthetic mixtures of triglycerides and of natural oils and fats on silver nitrate plates, using mixtures like carbon tetrachloride–chloroform–ethanol, was described by BARRET *et al.*[37,38].

Phosphatide mixtures *e.g.* from eggs were resolved on silica gel G–silver nitrate layers by KAUFMANN *et al.*[39] with the solvent mixture chloroform–ether–acetic acid (97.0:2.3:0.5).

Some poly-unsaturated olefins like humulene, caryophyllene, and thujopsene were separated on silica gel–silver nitrate plates by GUPTA AND SUKH DEV[40]. A fractionation of aldehyde 2,4-dinitrophenylhydrazones into "unsaturation classes" is described by URBACH[41], using aluminium oxide–silver nitrate plates, and by BADINGS AND WASSINK[42], employing kieselguhr G–silver nitrate chromatoplates. Since naturally occurring sterol mixtures are composed of saturated, mono-, di-, and poly-unsaturated sterols, it is to be expected that silver nitrate adsorbent layers will become a powerful tool in sterol and steroid analysis.

The analysis of the saturated dihydrocholesterol and the mono-unsaturated Δ^5-cholesterol in mutual mixtures incurs many difficulties, but because of their different degree of unsaturation dihydrocholesterol and cholesterol were readily separated on silica gel G–silver nitrate plates by DE VRIES, using chloroform or chloroform–acetic acid mixtures.

We have previously reported a clear resolution of these sterols in our reversed-phase system[5]. In this way small amounts of dihydrocholesterol (down to 5 %) were detected in an excess of cholesterol.

AVIGAN *et al.*[24] have briefly described the separation of cholestenols (Δ^5 and Δ^7), desmosterol, and 7-dehydrocholesterol on silica gel layers impregnated with $AgNO_3$ by spraying with a saturated $AgNO_3$ solution. A mixture benzene–ethyl acetate (5:1) was used as mobile phase. Cholesterol esters were fractionated by MORRIS[43] on silica gel G–silver nitrate plates with the solvent mixture ether–hexane (1:4), according to the unsaturation of the fatty acid chain.

In the present investigation we have studied the behaviour of several sterols and sterol acetates on silica gel G–silver nitrate layers more thoroughly.

Separation of sterols

We have followed the procedure of preparing silica gel G–silver nitrate chromatoplates described by DE VRIES[36] (see also KAUFMANN *et al.*[39]). 13 g of silver nitrate (Merck) were dissolved in 60 ml of water and added to 30 g of silica gel G (Merck); chromatoplates of 20 × 20 cm were then prepared from this mixture. After drying (for 2 h at 20° and then for 2 h at 110°) and spotting with 110–150 μg of sterols, the plates were developed in the solvent mixture chloroform–ether–acetic acid (97:2.3:0.5), according to KAUFMANN *et al.*[39].

After the development procedure the plates were sprayed with a 0.2 % ethanolic

solution of dibromofluorescein and viewed under U.V. radiation of 365 mμ. The sterol spots then showed a bright fluorescence. More details of the procedure are given in the experimental part.

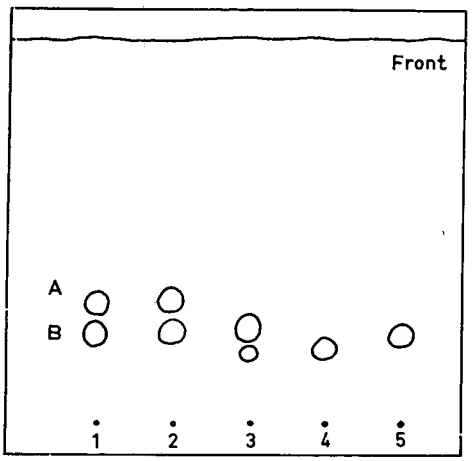

Fig. 3. TLC separation of sterols. Adsorbent: silica gel G–silver nitrate. Solvent: chloroform–ether–acetic acid (97:2.3:0.5). Time of run: 1.5–2 h. Detection: dibromofluorescein. Spot 1: 150 μg of a 1:1 mixture of dihydrocholesterol (A) and cholesterol (B); spot 2: 150 μg of a 1:1 mixture of Δ^7-cholestenol and cholesterol (B); spot 3: third band phytosterols isolated from coconut fat; spot 4: 80 μg ergosterol D; and spot 5: 80 μg stigmasterol.

The R_S values of several sterols in this system (A) are given in Table III. Besides separating dihydrocholesterol and cholesterol, this system enables the separation of several other pairs of closely related sterols *e.g.* cholesterol (FC_{27})–Δ^7-cholestenol (FC_{27}), cholesterol (FC_{27})–lanosterol ($FC_{30}F$), vitamin D_2 ($3FC_{28}F$)–dihydrovitamin D_2 ($3FC_{28}$), cholesterol (FC_{27})–"third-band phytosterols" ($FC_{28}F$), and 5-dihydroergosterol ($FC_{28}F$)–stigmasterol ($FC_{29}F$). Some of these sterol separations are shown in Fig. 3.

The R_S values of Table III and the chromatoplate of Fig. 3 indicate that in this system mono- and many di-unsaturated sterols have the same migration rate. Cholesterol, β-sitosterol, and stigmasterol show identical R_S values. Similarly, sterols having one double bond in the $\Delta^{7(8)}$ position like Δ^7-cholestenol, Δ^7-ergostenol, and 5-dihydroergosterol cannot be separated in this system.

Remarkably, these Δ^7-sterols form a critical pair with dihydrocholesterol and not with the corresponding Δ^5-sterol *viz.* cholesterol. The possibility of *separating Δ^5- and Δ^7-sterols* in this system is therefore of practical importance.

The triterpenoid alcohols lanosterol, agnosterol, and their 24-dihydro compounds show quite a high migration rate ($R_S \simeq 1.70$). Ergosterol D, *viz.* $\Delta^{7,9(11),22}$-ergostatrien-3β-ol ($2FC_{28}F$), with two conjugated double bonds has an R_S value of 0.83.

Sterols and steroids having a system of *three* conjugated double bonds like vitamins D_2 ($3FC_{28}F$) and $\Delta^{9(11)}$-dehydroergosterol ($3FC_{28}F$) have quite low migration rates *viz.* 0.64 and 0.69, respectively.

On account of their migration rates in the normal reversed-phase system and in the "bromine-system", the third-band phytosterols of *e.g.* coconut fat were ten-

TABLE III

SEPARATION OF STEROLS AND STEROL ACETATES ON SILICA GEL G–SILVER NITRATE LAYERS

System A: chloroform–ether–acetic acid (97:2.3:0.5); system B: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5).
Spotted amount: 50–100 μg.
Time of run: 1–2 h.
Detection: 0.2 % dibromofluorescein.

Compound	*Shorthand designation*	*R_s value of sterols in A*	*R_s value of sterol acetates in B*
Agnosterol	$2FC_{30}F$	1.68	0.40
24-Dihydroagnosterol	$2FC_{30}$	1.61	
Lanosterol	$FC_{30}F$	1.70	0.78
Dihydro-β-sitosterol	C_{29}	1.14	1.30
Dihydrocholesterol	C_{27}	1.14	1.25
Cholesterol	FC_{27}	≡ 1.00	≡ 1.00
β-Sitosterol	FC_{29}	1.00	1.00
Δ^7-Cholestenol	FC_{27}	1.17	1.14
Δ^7-Ergostenol	FC_{28}	1.22	1.21
5-Dihydroergosterol	$FC_{28}F$	1.13	0.88
Stigmasterol	$FC_{29}F$	0.98	0.87
Desmosterol	$FC_{27}F$	0.88	—
Vitamin D_2	$3FC_{28}F$	0.64	
Dihydrovitamin D_2	$3FC_{28}$	0.47	
Ergosterol D ($\Delta^{7,9(11),22}$-ergostatrienol	$2FC_{28}F$	0.83	
$\Delta^{9(11)}$-Dehydroergosterol	$3FC_{28}F$	0.69	
Brassicasterol	$FC_{28}F$	0.98(?)	0.68(?)
"Third-band phytosterols"	"$FC_{28}F$"?	0.87	0.33
Ergosterol	$2FC_{28}F$	0.44	0.35 *
7-Dehydrocholesterol	$2FC_{27}$	0.44	0.43 *

* After two developments; spotted amount 30 μg.

tatively supposed to have an $FC_{28}F$-sterol structure, slightly more polar than that of brassicasterol. The third-band phytosterols of coconut fat were isolated on a preparative scale from ten chromatoplates. After removing the contaminating undecane and after saponification of the sterol acetates, the residue was spotted on a silica gel G–silver nitrate plate. After the development procedure two spots appeared under U.V. radiation, a minor spot at $R_s = 1.05$ and a major one at $R_s = 0.87$. The latter spot has about the same migration rate as some poly-unsaturated sterols *e.g.* ergosterol D.

This experiment confirmed the conclusion, which was already drawn from the results in the bromine-system, that the polarity of the third-band phytosterols is somewhat higher than that of the isomerous brassicasterol.

In the bromine-system, only those sterols having a $\Delta^{5(6)}$ double band can be detected while all other sterol types are decomposed. Therefore, we may assume that one of the double bonds of the third-band phytosterols should be at $\Delta^{5(6)}$, while the special position of the other one may cause the relatively high polarity of the molecule[5]. In this respect we tentatively might consider a double bond situated nearer to the terminal alkyl groups *e.g.* $\Delta^{24(28)}$ ($\Delta^{5,24(28)}$-ergostadienol), $\Delta^{24(25)}$ (desmosterol) or $\Delta^{25(26)}$. Such positions are known to cause longer retention times in the gas chromato-

graphic analysis of fatty acids (ACKMAN AND BURGHER[44]) and sterols (RECOURT AND BEERTHUIS[45]). Since we did not succeed in resolving mono- and di-unsaturated sterols like cholesterol and stigmasterol in this system, it was supposed[46] that the free hydroxyl group exerts too great an influence on the adsorption of the sterols. It seemed worthwhile to study the separation of the less polar sterol acetates.

Separation of sterol acetates

After testing various solvent mixtures the best results for fractionating mixtures of sterol acetates on silica gel G–silver nitrate layers were obtained when using the mobile phase B: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5). In this system still better separations of the groups of saturated, mono-, di-, and polyunsaturated sterols may be accomplished. The R_s values of a number of sterols in this system *viz.* B are given in Table III.

Although the acetates of cholesterol and stigmasterol have slightly different migration rates in this system their separation is only possible under favourable conditions *e.g.* at a spotted amount of only 30 μg of the mixture (see Table III).

The triterpenoid alcohols, lanosterol and agnosterol were clearly separated, analogous to the investigations of DEN BOER[46].

In contrast to the results of system A, in system B the spot of Δ^7-cholestenol does not coincide with that of dihydrocholesterol, but has an R_s value intermediate to those of dihydrocholesterol and cholesterol. Under favourable conditions it is possible to resolve the spots of Δ^7-cholestenol and Δ^7-ergostenol.

This system enables a clear separation between the acetates of Δ^7-cholestenol (or Δ^7-ergostenol) and the di-unsaturated 5-dihydroergosterol, whereas the corresponding sterols have identical R_s values in system A. The sterol mixture from the starfish *Asterina pectinifera*, which according to TOYAMA contains Δ^7-cholestenol,

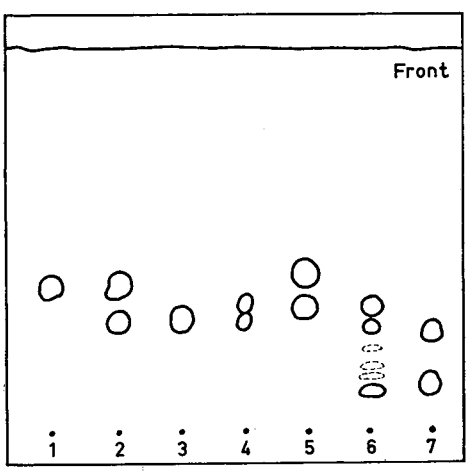

Fig. 4. TLC separation of sterol acetates. Adsorbent: silica gel G–silver nitrate. Solvent: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5). Time of run: 1–2 h. Detection: dibromofluorescein. Spot 1: 100 μg Δ^7-cholestenol acetate; spot 2: 150 μg of the acetates of *Asterina pectinifera* sterols; spot 3: 100 μg 5-dihydroergosterol; spot 4: 30 μg of a 1:1 mixture of cholesterol acetate and stigmasterol acetate; spot 5: 200 μg of a 1:1 mixture of dihydrocholesterol acetate and cholesterol acetate; spot 6: 60 μg of the phytosterol acetates from coconut fat; and spot 7: 100 μg of a 1:1 mixture of lanosterol acetate and agnosterol acetate.

$\Delta^{7,22}$-C_{28} sterol, α-spinasterol and Δ^{7}-stigmastenol, was thus fractioned into two major spots at R_s values of 0.88 and 1.08. These spots are most likely attributed to the acetates of Δ^{7}-cholestenol and 5-dihydroergosterol (see Fig. 4, spot 2). Furthermore, two minor spots at lower R_s values *viz.* 0.48 and 0.29 were visible when spotting 200 μg of the sterol acetate mixtures. Most probably, these minor spots are due to "polar" sterols, causing the fourth band at $R_s = 1.22$ in the normal reversed-phase system (see Fig. 1, spot 8).

In spite of their low migration rates the acetates of the critical-pair partners 7-dehydrocholesterol and ergosterol are separable in system B. In general, in this system the acetates of "polar" sterols like these provitamins D and triterpenoid alcohols, *e.g.* agnosterol, have quite low migration rates.

Using system B the phytosterol mixtures isolated from various edible oils and fats can be fractionated into several distinct spots. An amount of 60 μg of the acetates of the phytosterols isolated from coconut fat is fractionated in this way into two clearly separated spots at $R_s = 1.00$ and $R_s = 0.88$, caused by β-sitosterol and stigmasterol acetate, respectively. Furthermore, three weak fluorescent spots are visible at R_s values of 0.68, 0.49 and 0.44, respectively, and a relatively large spot at $R_s = 0.33$, due to the "third-band phytosterols" (see Fig. 4, spot 6).

The identity of the third-band phytosterol spot was deduced among other things from the study of the acetates of the phytosterols from almond oil, which only exhibit a β-sitosterol band in the undecane/acetic acid–acetonitrile (1:3) system. Besides a β-sitosterol and a "third" band at $R_s = 1.00$, on silica gel G–silver nitrate plates only one other band (at $R_s = 0.33$) is shown, thus identifying itself with the third-band phytosterols in the reversed-phase system.

EXPERIMENTAL

Reversed-phase system

A volume of about 400 ml of the solvent mixture acetic acid–acetonitrile (1:3) is saturated with 18 ml of undecane* by shaking both phases in a separatory funnel. After a settling time of 16 h at 22–23° both layers are separated. The undecane is diluted with light petroleum (b.p. 40–60°) to a 10 % solution; the latter is used for the impregnation procedure.

The acetic acid–acetonitrile mixture is introduced into a small chromatographic vessel of about 19 × 30 × 7 cm, which is lined with filter paper on all sides to ensure complete saturation. The vessel is equilibrated at 22–23° for 24 h.

Glass plates of 14 × 24 cm are coated with a kieselguhr G (Merck)–water (1:2)-mixture, according to the procedure of STAHL. The 0.24–0.28 mm layers are activated by heating at 100° for 15 min. After cooling to room temperature, the layers are impregnated with the 10 % undecane solution. The chromatoplate is taken between thumb and forefinger of both hands (wearing rubber gloves) and is dipped carefully into a shallow tray containing this solution. Care must be taken that the layer is not damaged. After the impregnation procedure, lasting only a few seconds, the plate is held upside down for some ten seconds to remove the excess of undecane solution. The plate is then stored at room temperature on a horizontal surface for a standard

* Undecane standardized, b.p. 190–220°, $n_D^{20} = 1.4307$, available from J. HALTERMANN, Hamburg.

evaporation period. The duration of this period depends on the thickness of the layer, dimensions of the plate, temperature of the room etc. In our case a period of 80 min was selected, after which time 0.19 g of undecane was left on the plate *viz.* 0.04 g/g of kieselguhr G*.

During this evaporation period hexagonal** pieces (of 20 × 23 mm) are removed from the layer with a brush, using an appropriate template. In this way 4 chromatograms are modelled. The distance of the centre of the 8 mm wide "bridges" to the bottom of the plate is 40 mm. At the end of the evaporation period the sterol acetate solution (preferably 15 mm^3 of a 0.2 % ethereal solution) is spotted at that centre by means of a micro pipette.

The chromatoplate is then developed in the chromatographic vessel. When the solvent front has travelled 20 cm in some 1.5 h, the development is discontinued and the plate is dried in air for 2–4 h and afterwards for 45 min at 100°.

The plate is sprayed with a 20 % ethanolic solution of phosphomolybdic acid (Merck) and heated for 5–10 min at 100° till the bands are coloured to the maximum intensity. After each development procedure, the solvent mixture must be discarded and a freshly prepared mixture should be used.

"Bromine-system"

In this bromine-system nearly the same procedure applies. The temperature during development should preferably be 18–20°. Prior to the development procedure 0.5 % of bromine is added *within* the chromatographic vessel. The developed chromatoplate is dried in air for 2 h and then for some 10 min at 100°. Generally, during the heating bright blue bands appear. The bands of brassicasterol and of the FC_{28}F-sterols are coloured faintly. To increase the colour intensity the warm plate is afterwards sprayed with a 50 % solution of antimony(III) chloride in acetic acid or with an acetic anhydride–sulfuric acid 50 % (1:2) mixture. The colour of the bands is now intensified, especially those of brassicasterol and FC_{28}F-sterols, which attain a violet shade. The bands of the other sterols are coloured bright blue.

"Silver nitrate layers"

13 g of silver nitrate (Merck) is dissolved in 60 ml of water and 30 g of silica gel G (Merck) is admixed. The mixture is spread upon 4 chromatoplates of 20 × 20 cm. The plates are dried in the dark for 2 h and then activated for 1 h at 110°. After spotting 100–200 μg of the ethereal solutions of the sterols or their acetates, the plate is developed with either the solvent mixture A, chloroform–ether–acetic acid (97:2.3:0.5), or B, chloroform–light petroleum–acetic acid (25:75:0.5). The chromatographic vessel is lined with filter paper which is saturated with the solvent mixture and equilibrated for some 16 h. After the development procedure, the chromatoplate is dried in air and then sprayed with a 0.2 % solution of dibromofluorescein in 96 % ethanol. The plate is then viewed under U.V. radiation of 365 mμ and the fluorescent spots are marked with a pencil.

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* On the common 20 × 20 cm "Desaga" plates with layers of about 0.18 mm, a pentagonal figure with its base at 10 mm distance from the bottom of the plate is modelled. In this case an evaporation period of 60 min in air is employed (degree of impregnation 0.08 g/g). Length of run 17 cm.

** Pentagonal figures may also be used.

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SUMMARY

The analysis of sterols by means of thin-layer chromatography (TLC) is investigated. Previous experiments of the authors on the reversed-phase system undecane/acetic acid–acetonitrile (1:3) are amplified. For identification purposes the sterols are characterised by their so-called carbon numbers.

By adding bromine to the mobile phase several critical pairs of sterols were resolved. The properties of this "bromine system" are discussed.

On silica gel G–silver nitrate layers the sterols or their acetates are separated according to their degree of unsaturation. The procedures for this $AgNO_3$ TLC, which enable a clear separation of many sterols, are described in detail. The above chromatographic analyses can be applied to the identification of unknown sterols isolated from natural sources, *e.g.* coconut fat and *Asterina pectinifera*.

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THIN-LAYER CHROMATOGRAPHY OF CINCHONA ALKALOIDS

II. QUALITATIVE EXAMINATION OF DIHYDRO-BASES OF THE CINCHONA ALKALOIDS IN COMMERCIAL PRODUCTS

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Commercial preparations of quinine, quinidine, cinchonine and cinchonidine obtained on a technical scale from cinchona bark are not pure compounds but contain small amounts of other vinylic bases and their dihydro derivatives[1]. The constituents vary according to the species of tree and the technology of separation and preparation.

It was felt that the non-uniformity of these substances made it necessary to have a rapid and effective method of identification and qualitative assay of the impurities with regard to synthetic and analytical research work and pharmaceutical applications. Such a method could also serve for establishing new commercial standards for the production of these alkaloids and for the pharmaceutical industry.

Consequently, we attempted to apply earlier results[2] obtained by thin-layer chromatography to the qualitative assessment of alkaloid preparations from cinchona bark. In particular, this paper is aimed at presenting results on the content of dihydro-bases in various commercial preparations of the alkaloids under consideration.

EXPERIMENTAL

The following commercial preparations of quinine, quinidine, cinchonine and cinchonidine were examined:

(1) Quinine (Toscat Brand, Briddle Sawyer and Co. Ltd., London, Great Britain).

(2) Quinine hydrochloride (Toscat Brand, Briddle Sawyer and Co. Ltd., London, Great Britain).

(3) Quinine hydrochloride (N.V. Amsterdamsche Chininefabriek, Amsterdam, The Netherlands).

(4) Quinidine (E. Merck, Darmstadt, Germany).

(5) Quinidine (BDH Laboratory Reagent, The British Drug Houses Ltd., BDH Laboratory Chemical Group, Poole, Great Britain).

(6) Quinidine (Toscat Brand, Briddle Sawyer and Co. Ltd., London, Great Britain).

(7) Quinidine (N.V. Amsterdamsche Chininefabriek, Amsterdam, The Netherlands).

(8) Quinidine sulfate (N.V. Amsterdamsche Chininefabriek, Amsterdam, The Netherlands).

(9) Cinchonine (BDH Laboratory Reagent, The British Drug Houses Ltd., BDH Laboratory Chemical Group, Poole, Great Britain).

(10) Cinchonine (N.V. Amsterdamsche Chininefabriek, Amsterdam, The Netherlands).

(11) Cinchonine hydrochloride (Toscat Brand, Briddle Sawyer and Co., Ltd., London, Great Britain).

(12) Cinchonidine (BDH Laboratory Reagent, The British Drug Houses, Ltd., BDH Laboratory Chemical Group, Poole, Great Britain).

(13) Cinchonidine (W. Dembach and Co., Bad Ems, Germany).

(14) Cinchonidine (C. F. Boehringer and Soehne, GmbH, Mannheim, Germany).

Standards were obtained by preparative purification[2] of the purest commercial raw materials of cinchona bark alkaloids.

Preparation of the plates with adsorbent and development of the chromatograms were carried out according to the method already described[2]. Five per cent solutions in butyl alcohol of the standard substances and the commercial samples under investigation were obtained by dissolving 50 mg of the substance in 10 ml volumetric flasks. Single applications of the substances were made by means of a calibrated platinum wire loop, 2.4 μg of the alkaloid being applied to the 10 $\times$ 20 cm plates used. Two chromatograms were run simultaneously in the same chamber, providing for identical conditions for diastereoisomers. They were dried in a current of warm air and observed under a quartz lamp with filter to assess the fluorescent intensity of the spots corresponding to the hydro-bases. The centre of intensity was determined and the chromatograms were sprayed with Dragendorff's reagent according to MUNIER[3] after which observation was repeated in U.V. light. When the layer had dried, the relative sizes of the coloured spots and their colorations were determined.

Calibration of the wire was effected with respect to butyl alcohol, which was used as solvent for the standards and samples under investigation. From a volume of butanol whose weight had been determined to within 0.1 mg, sampling of the solvent with the wire was repeated 100 times, spreading the amount taken on paper or on a plate and weighing again. From the difference, the weight of a single loop probe was calculated as 0.388 mg, whence the volume of the platinum wire used was obtained in μl (0.48).

RESULTS AND DISCUSSION

In a series of experiments, the commercial preparations of cinchona bark alkaloids available were investigated for their dihydrobase content.

Almost all the samples were found to contain impurities whose R_F corresponded to the R_F values of the standard hydro-bases.

From the intensity of the fluorescence, size and coloration of the spots developed with the reagent, information could be obtained as to the quality of the preparation. Observation corroborated qualitatively data from the literature[1] relating to the hydro-base content in quinidine and cinchonine as determined by other methods[4]. Preparations of these two alkaloids (Figs. 2 and 3) revealed the highest hydrobase content, the latter attaining its highest value in quinidine.

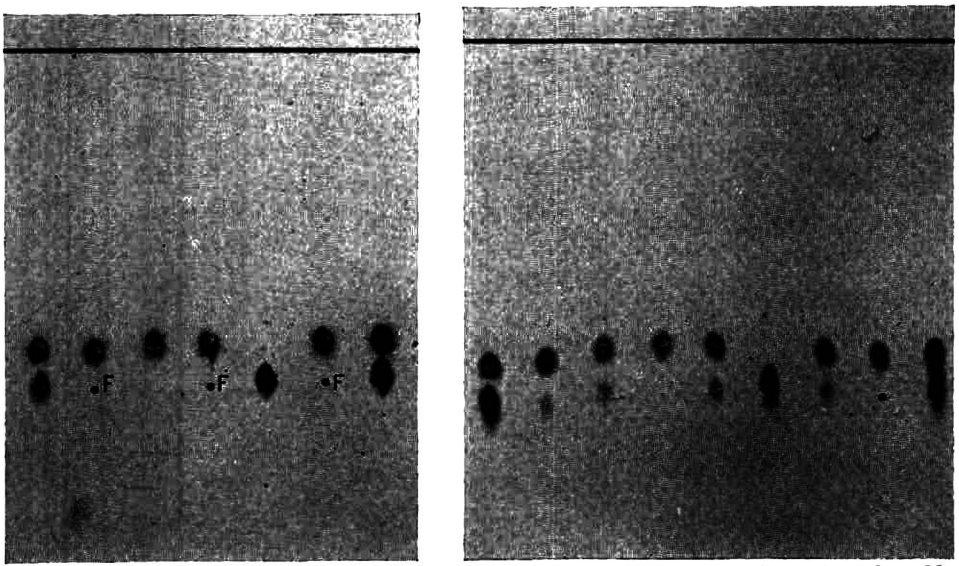

Fig. 1. Thin-layer chromatogram of commercial quinine 1, 2, 3. M_1 = mixture of quinine and dihydroquinine; S_1 = quinine; S_2 = dihydroquinine; F = centre of fluorescence of dihydroquinine in commercial quinine.

Fig. 2. Thin-layer chromatogram of commercial quinidine 4, 5, 6, 7, 8. M_2 = mixture of quinidine and dihydroquinidine; S_3 = quinidine; S_4 = dihydroquinidine.

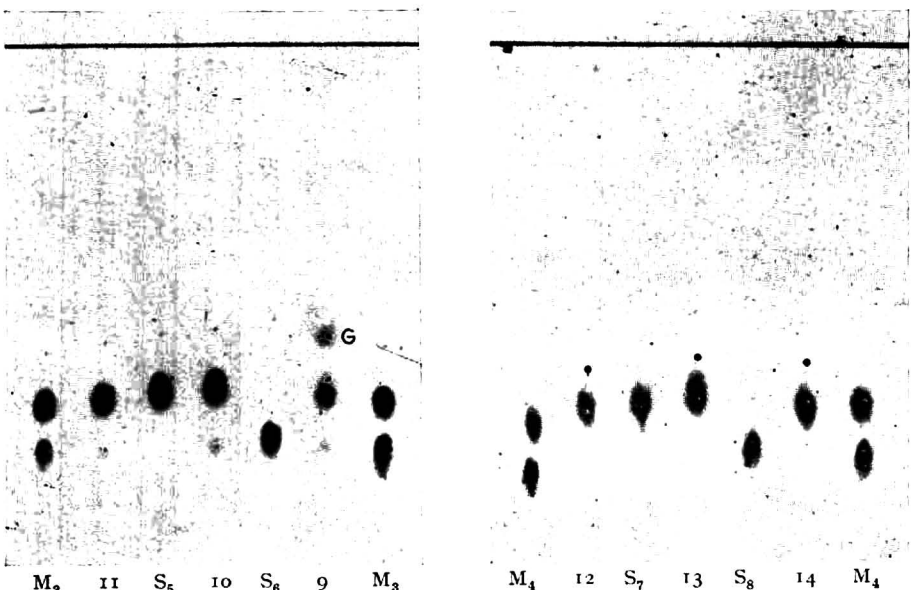

Fig. 3. Thin-layer chromatogram of commercial cinchonine 9, 10, 11. M_3 = mixture of cinchonine and dihydrocinchonine; S_5 = dihydrocinchonine; S_6 = dihydrocinchonine; G = additional component contaminating cinchonine hydrochloride.

Fig. 4. Thin-layer chromatogram of commercial cinchonidine 12, 13, 14. M_4 = mixture of cinchonidine and dihydrocinchonidine; S_7 = cinchonidine; S_8 = dihydrocinchonidine.

The technique of spotting with a calibrated platinum wire loop and a 25 min run produced qualitative results in a relatively short time.

Since butanol is a solvent in which all alkaloids of this group dissolve in the concentrations used, equal standards could be obtained. Moreover, butanol solutions of the alkaloids are well adapted to storage. The lower volatility of butanol as compared with methanol or chloroform provided for more accurate spotting of well-determined concentrations of the substances on the plates. Comparability of the results hinges on the accuracy achieved in preparing and spotting the solutions of the samples for investigation.

Preparations of salts of the alkaloids were found to be of higher purity than the free bases (Fig. 2, preparation 8).

Quinine

Dihydro-base impurities in commercial quinine preparations (Fig. 1) were detected primarily from their fluorescence and verified by the coloration (hardly visible at such concentrations) of the spot on spraying with Dragendorff's reagent. ΔR_F's for the mixture of standards and ΔR_F's for the preparations investigated amounted to 0.07–0.08. Qualitative chromatographic analysis of the quinine samples showed that dihydro-base fluorescence in the preparation of quinine hydrochloride F 3 was the weakest. More detailed observation showed that sample 3 had the lowest hydro-base content, whereas the free base preparation 1 had the highest (Table I).

TABLE I

ΔR_F AND R_F VALUES OF CINCHONA BARK ALKALOIDS IN COMMERCIAL PREPARATIONS, AND COMPARATIVE ASSESSMENT OF SAMPLES OF COMMERCIAL QUININE, QUINIDINE, CINCHONINE AND CINCHONIDINE

The numbers of the samples are those in the list in the text.

No.	*R_F of the dihydro-base*	*R_F of the vinyl-base*	*ΔR_F*	*Dihydro-base content in the commercial preparation*
1	0.42	0.50	0.08	1 > 2 > 3
2	0.41	0.49	0.08	max. min.
3	0.42	0.50	0.08	
4	0.36	0.44	0.08	6 > 7 > 5 > 4 > 8
5	0.38	0.46	0.08	max. min.
6	0.39	0.47	0.08	
7	0.38	0.46	0.08	
8	0.37	0.45	0.08	
9	0.29	0.38	0.09	10 > 9 > 11
10	0.30	0.40	0.10	max. min.
11	0.29	0.39	0.10	
12	—	0.36	0.43	
13	—	0.39	0.46	
14	—	0.37	0.46	

Kieselgel G Merck 7731. Solvent system: chloroform–methanol–diethylamine (80:20:1). Solvent front: 10 cm. The alkaloids were dissolved in butanol. 2.4 μg of alkaloid was applied to the plate. Time of run: 25 min. Spray reagent: Dragendorff by MUNIER[3].

Quinidine

In the case of quinidine (Fig. 2), the preparation of quinidine sulfate 8 showed the weakest hydro-base fluorescence with only weakly visible coloration of the spots, pointing to the lowest dihydroquinidine content. On the basis of the classification criteria assumed, the other samples were found to contain varying amounts of the latter, the quinidine preparation 6 containing the greatest amount of the dihydro-base while quinidine sulfate 8 had only traces (Table I).

In the case of the quinidine preparations, ΔR_F for separation of the base and its hydro derivative amounted to 0.08.

Cinchonine

Chromatographic analysis of cinchonine preparations (Fig. 3) revealed that cinchonine 10 contained the greatest amount of dihydro-base contamination, whereas the hydrochloride 11 had only traces (Table I).

It is noteworthy, however, that the above preparation of cinchonine hydrochloride contained yet another impurity giving an intense fluorescence and being intensely colored after spraying with the developing reagent. Its R_F value was found to be 0.49.

For the cinchonine preparations, ΔR_F for separation of the base and hydro-base ranged between 0.09 and 0.10 (Table I).

Cinchonidine

The cinchonidine preparations 12, 13 and 14 (Fig. 4) at the concentrations employed for spotting revealed no dihydro-derivatives. However, the preparations of this alkaloid gave a spot which fluoresced in U.V. light at an R_F value of 0.43–0.46 (Table I), pointing to the presence of an additional impurity. Similar fluorescence was found in some of the other samples dealt with. However, the problem requires further investigation.

The above experimental results show that qualitative chromatographic analysis on thin layers permits rapid detection of dihydroalkaloids and a comparative evaluation of their content in commercial preparations of cinchona bark.

The method as elaborated above has been applied by one of the authors for controlling the process of purification of commercial cinchona bark preparations by the THRON AND DIRSCHERL method[5].

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The authors wish to thank Prof. Dr. J. SUSZKO for making available the commercial preparations of the alkaloids.

SUMMARY

Fourteen commercial preparations of cinchona bark alkaloids were tested for their hydro-base content by thin-layer chromatography. From the intensity of fluorescence as well as the size and intensity of coloration of the spots, the content of dihydro-bases was assessed, thus establishing the quality of the commercial preparation in each case.

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THE FLUORESCENCE-QUENCHING EFFECT IN THIN-LAYER CHROMATOGRAPHY OF POLYNUCLEAR AROMATIC HYDROCARBONS AND THEIR AZA ANALOGS

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INTRODUCTION

Polynuclear aromatic hydrocarbons show vivid fluorescence on thin-layer chromatograms[1,2]. The detection limits range from about 1 to 1000 ng and average[2] about 10 ng. Consequently the fluorescent colors are of practical value in separating trace amounts of these compounds from extremely complicated mixtures, such as organic airborne particulates. Other polynuclear aromatic compounds, such as aldehydes, ketones, amines, and oxygen, sulfur, aza and imino heterocyclic compounds also show fluorescent colors of the same order of intensity on thin-layer chromatograms[2]. The detection limits for the aza heterocyclic compounds average around 3 ng, and most of the compounds can be detected at 1 ng or 0.001 μg.

It was believed that since the polynuclear compounds are so intensely fluorescent, any method that could selectively quench some of these groups of compounds would be invaluable in group characterization through thin-layer and paper chromatographic studies. Since organic atmospheric and air pollution source particulates contain an extremely large number of unknown fluorescent molecules[3], the quenching effect should help in the characterization of these unknowns.

EXPERIMENTAL

Materials

All polynuclear aromatic compounds were obtained from commercial sources.

Equipment

The fluorescent colors were examined and sprayed in a Chromato-Vue cabinet (Kensington Scientific Corp., Berkeley 10, Calif.) under a 3660 Å light source.

Solvent systems

Pentane–ether (19:1, v/v); pentane–2-nitropropane (19:1, v/v); pentane–2-nitropropane (9:1, v/v); pentane–nitrobenzene (9:1, v/v).

Fuming reagents

Trifluoroacetic acid, nitromethane, carbon disulfide.

Method of development

Smooth glass plates (20 × 20 cm) were coated with 250 μ thickness of alumina with an applicator. The development was performed by the ascending method in the usual procedure.

Fuming methods

Trifluoroacetic acid test. The line of development is treated with small bursts of trifluoroacetic acid fumes. A throwaway pipet fitted with a squeeze bulb or a closed tank containing the plate and trifluoroacetic acid fumes can be used. Any change in fluorescence color is noted.

Other fuming reagents used in the procedure were nitromethane, carbon disulfide, and nitrogen dioxide–trifluoroacetic acid (3:10). Nitromethane works best as a fine liquid spray.

DISCUSSION

Thin-layer chromatography of aza compounds

One factor common to all the separations of aza compounds on alumina, whether with pentane–nitrobenzene (9:1, v/v) or pentane–2-nitropropane (3:1, v/v), is that these substances are separated according to their size and the amount of steric hindrance around the aza nitrogen atom. The adsorbent has a very strong attraction for the aza nitrogen atom. For this reason the following isomers are readily separated: benzo(*f*)quinoline and benzo(*h*)quinoline, benz(*a*)acridine and benz(*c*)acridine, 7-phenyldibenz(*c,h*)acridine and 14-phenyldibenz(*a,j*)acridine. Characteristics of these and other compounds are presented in Table I. The R_F values varied with

TABLE I

R_F VALUES AND FLUORESCENCE COLORS OF SOME AZA HETEROCYCLIC COMPOUNDS ON ALUMINA

Compound	*Pentane–nitrobenzene (9:1, v/v)*			*Pentane–2-nitropropane–triethylamine (9:1:0.01)*			
	R_F	*Color**		R_F	*Color**		
		Wet plate	*After TFA*		*Wet plate*	*Dry plate*	*After TFA*
7-Phenyldibenz(*c,h*)acridine	0.94	—**	YG	0.93	BG	BG	YG
Benz(*c*)acridine	0.92	—	G	0.92	—	B	G
Pyrenoline	0.45	—	RO	0.82	B	B	RO
Acridine	0.37	lG	G	0.66	—	B	G
Benzo(*h*)quinoline	0.35	—	B	0.90	—	B	B
Benzo(*f*)quinoline	0.30	—	B	0.53	—	B	B
14-Phenyldibenz(*a,j*)acridine	0.28	G	G	0.67	B	B	B
Indeno(1,2,3-*i,j*)isoquinoline	0.26	lB	dRO	0.65	YG	YG	R
Benz(*a*)acridine	0.23	lG	BG	0.61	B	B	BG
Acenaphtho(1,2-*b*)pyridine	—	—	—	0.59	B	B	BG
Benzo(*l,m,n*)phenanthridine	0.15	—	G	0.48	B	B	G

* B = blue; G = green; O = orange; Pk = pink; R = red; Y = yellow; d = dull; l = light.
** — = quenched.

changes in the amount of water on the alumina and with varying batches of alumina. Standards should be run automatically on the plate with any unknown material that is being investigated.

Pyrenoline is readily detected on the plate after separation followed by about 5 min of irradiation under the 3660 Å light source of the Chromato-Vue cabinet. A visible violet color is obtained, with a detection limit of ~0.01 μg of pyrenoline. Acid interferes in this characterization test. Thus, through the ordinary procedure of separation and examination of the plate under ultraviolet light, pyrenoline can be selectively differentiated from all the other aza heterocyclic compounds.

Direct quenching

With a developing mixture consisting of pentane–nitrobenzene (9:1, v/v), aromatic hydrocarbons are separated near the top of the plate but cannot be seen under ultraviolet light because their fluorescence has been quenched. Compounds such as anthracene, phenanthrene, pyrene, chrysene, fluoranthene, benz(*a*)anthracene, benzofluorenes, benzopyrenes, perylene, benzo(*k*)fluoranthene, anthanthrene, and dibenzopyrenes are non-fluorescent on such a plate. In addition benzo(*f*)quinoline, benzo(*h*)quinoline, pyrenoline, and 7-phenyldibenz(*c*,*h*)acridine are quenched. At higher concentrations the latter compound will appear as a blue-green fluorescent

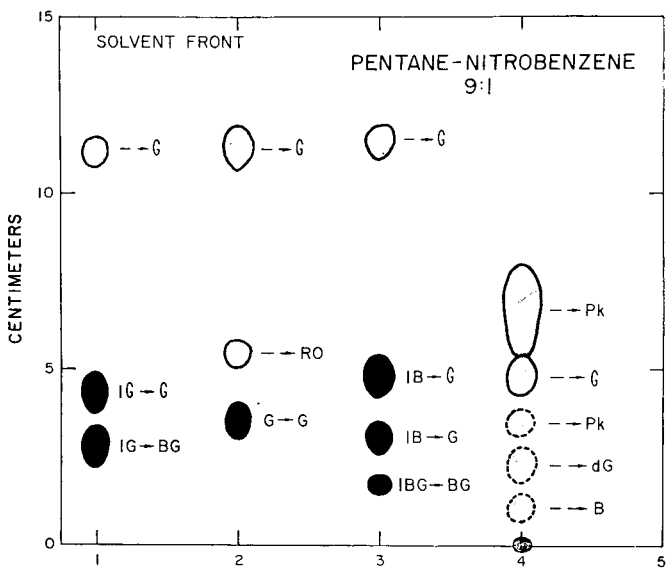

Fig. 1. Thin-layer chromatogram on alumina with pentane–nitrobenzene (9:1, v/v) as developer. All compounds were present in 2-μg amounts. Reading from top to bottom: (1) Benz(*c*)acridine, acridine and benz(*a*)acridine. (2) 7-Phenyldibenz(*c*,*h*)acridine, pyrenoline and 14-phenyldibenz(*a*,*j*)acridine. (3) A basic fraction obtained from an airborne particulate sample in which the main source of pollution was coal tar pitch fumes. (4) A benzene-soluble fraction of urban airborne particulates. The notation — ⟶ G signifies that the compound was not fluorescent on the wet plate but on treatment with trifluoroacetic acid fumes it fluoresced with a green color. The following compounds were also separated on this plate and were non-fluorescent on the wet plate or after treatment with trifluoroacetic acid fumes: anthracene, phenanthrene, pyrene, benzo(*a*)pyrene, benzo(*e*)pyrene, fluoranthene, benzo(*k*)fluoranthene and acenaphtho(1,2-*b*)pyridine. For color abbreviations see footnote * to Table I.

spot. The last two compounds become fluorescent when they are treated with trifluoroacetic acid fumes. An example of such a separation is shown in Fig. 1. The polycyclic aromatic hydrocarbons do not show under ultraviolet light, whereas most of the aza heterocyclic derivatives do. With trifluoroacetic acid fumes some of the non-fluorescent aza heterocyclic compounds become fluorescent. Thus by this system mixtures containing both aromatic and aza hydrocarbons can be readily separated and the latter derivatives can be more readily characterized. Some of the other types of aromatic compounds that have been found to be non-fluorescent in this type of system are 1-aminopyrene, 2-aminoanthracene, carbazole, the benzocarbazoles, 1-acetylpyrene, salicylaldehyde and benzanthrone. This system of separation and characterization appears to be highly selective for many of the larger aza heterocyclic hydrocarbons.

A second way of taking advantage of the quenching effect in thin-layer chro-

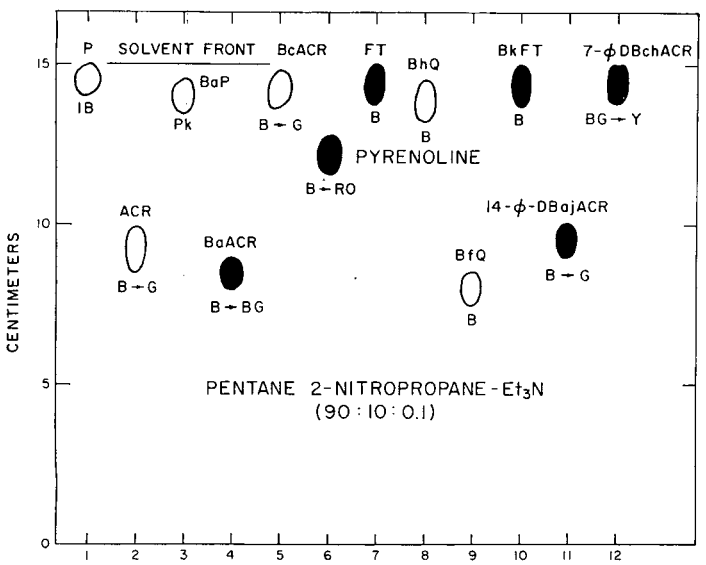

Fig. 2. Thin-layer chromatogram on alumina with pentane–2-nitropropane–triethylamine (9:1:0.01, v/v) as developer. (1) pyrene; (2) acridine; (3) benzo(*a*)pyrene; (4) benz(*a*)acridine; (5) benz(*c*)acridine; (6) pyrenoline; (7) fluoranthene; (8) benzo(*h*)quinoline; (9) benzo(*f*)quinoline; (10) benzo(*k*)fluoranthene; (11) 14-phenyldibenz(*a,j*)acridine; (12) 7-phenyldibenz(c_7h)acridine. Darkened spots are fluorescent on the wet or dry plate. Light spots are non-fluorescent on the wet plate and become fluorescent after approximately 1 h of standing. Spots on dry plate show no change in fluorescent color with trifluoroacetic acid fumes except as shown after an arrow. For color abbreviations see footnote * to Table I.

matography is by using a low-boiling quenching solvent in the developer. The evaporation should be just fast enough that the plate will be wet with this quencher for about half an hour. Two readings of the plate are made: one while it is wet and one while it is dry. In this way the quenched and non-quenched spots can be differentiated and found on the plate. An example of this type of differentiation is shown in Fig. 2. Non-fluoranthenic polycyclic hydrocarbons are non-fluorescent on a fresh plate, *e.g.* anthracene, pyrene, chrysene, the benzopyrenes, perylene, anthanthrene,

etc. Fluoranthenic hydrocarbons such as fluoranthene and benzo(*b*)- and benzo(*k*)-fluoranthene are fluorescent on the wet or dry plate. Some of the aza heterocyclic hydrocarbons are fluorescent and some are not. Once the plate is dry, however, all spots fluoresce. Trifluoroacetic acid fumes can bring out the fluorescence of all the aza compounds on the fresh plate.

For this kind of system the developer is an alkane containing 5–10 % of a nitroalkane. If a little acid is present in the developing solution instead of the triethylamine, then development is somewhat similar but all aza and fluoranthenic compounds are fluorescent on the wet plate under ultraviolet light.

Indirect quenching

A third fluorescence quenching method of value in thin-layer chromatography consists of treating a chromatogram with the fumes of a low boiling-quencher, *e.g.*

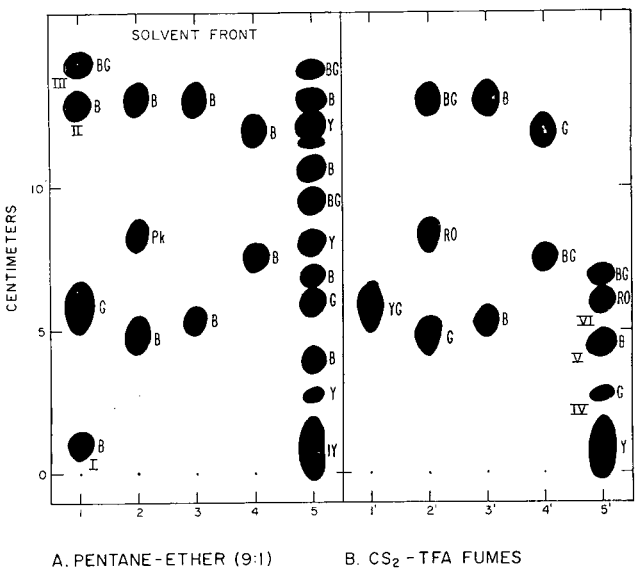

Fig. 3. Thin-layer chromatogram on alumina with pentane–ether (9:1, v/v) as developer. All compounds were present in approximately 2 μg amounts. Reading from top to bottom: (1) Pyrene, benzo(*a*)pyrene, benzanthrone and 1-aminopyrene. (2) Fluoranthene, pyrenoline and benz(*a*)-acridine. (3) 7-Phenyldibenz(*c,h*)acridine and benzo(*f*)quinoline. (4) Benz(*c*)acridine and acridine. (5) Benzene-soluble fraction of urban airborne particulates. (A) Read under ultraviolet light after development; (B) then spray with fumes of carbon disulfide saturated with trifluoroacetic acid. For color abbreviations see footnote * to Table I.

nitromethane, carbon disulfide or nitrogen dioxide. An example of this quenching effect is shown in Fig. 3. Here the quenching fumes consist of carbon disulfide saturated with trifluoroacetic acid. Compounds such as 1-aminopyrene (I), benzo(*a*)pyrene (II) and pyrene (III) are quenched. Carbon disulfide is a more potent quencher, for in addition to quenching spots I, II and III it also quenches benzo(*f*)quinoline. Nitromethane spray or nitrogen dioxide–trifluoroacetic acid fumes will also quench many non-fluoranthenic polycyclic aromatic hydrocarbons. With a nitroalkane or a carbon disulfide quencher the fluorescence of non-fluoranthenic hydrocarbons, polycyclic

carbazoles, many polycyclic aromatic amines, and many aromatic carbonyl derivatives can be quenched on a thin-layer plate. It is even possible to quench some of the aza heterocyclic compounds on the plate with freshly distilled nitromethane. Thus, acridine, the benzacridines and pyrenoline can be quenched while the fluoranthenic hydrocarbons still fluoresce. For this quenching to take place, the spot must be saturated only with nitromethane, and acid must be absent.

With the help of trifluoroacetic acid fumes all the aza compounds can be made to fluoresce as salts. Thus, the aza compounds can be distinguished from the fluoranthenic hydrocarbons, since with acid they become fluorescent, their fluorescence intensifies, or the fluorescent color changes. Additional evidence by which an aza hydrocarbon can be distinguished from an aromatic hydrocarbon is obtained by fuming the spots with nitrogen dioxide–trifluoroacetic acid (7:3, v/v) fumes. The aromatic hydrocarbons are quenched, while most of the aza hydrocarbons remain fluorescent. For example, pyrene, fluoranthene, benzo(*a*)pyrene, perylene and benzo(*k*)fluoranthene are quenched while indeno(1,2,3-*i*,*j*)isoquinoline, dibenz(*a*,*j*)acridine and benzanthrone have dull yellow-brown, dull gray-blue and dull orange-yellow fluorescence, respectively. Acridine, benzo(*f*)quinoline, benzo(*h*)quinoline, benz(*a*)-acridine, benz(*c*)acridine, acenaphtho(1,2-*b*)pyridine, benzo(*l*,*m*,*n*)phenanthridine and pyrenoline are brilliantly fluorescent under these conditions. On the basis of the work in this paper it is believed that highly selective quenchers for diverse groups of compounds can be developed and should prove extremely useful in paper and thin-layer chromatographic separation of fluorescent compounds.

APPLICATION

The separation of a basic fraction obtained from polluted air is shown in Fig. 1. The presence of aza compounds can be inferred from the presence of the fluorescent spots. Work on the identification of these spots is proceeding.

The separation of a benzene-soluble fraction of an urban airborne particulate sample is shown in Fig. 3A. The majority of the spots are quenched with carbon disulfide–trifluoroacetic acid. A new spot, V, appears in Fig. 3B. The emerald green spot at IV and the red-orange spot at VI are common to the organic benzene-soluble fractions of urban airborne particulates. These basic compounds are invaluable as markers.

SUMMARY

Fluorescence quenching effects were studied with volatile quencher in the developer, with a relatively non-volatile quencher in the developer, and with a volatile quencher after development. On the basis of quenching effects the aromatic compounds can be divided for analytical purposes into non-fluoranthenic aromatic, fluoranthenic and aza hydrocarbons. The latter group can be further subdivided.

Two systems for the separation of polycyclic aza hydrocarbons elute according to the size of the ring system and the amount of steric hindrance at a ring nitrogen atom.

Pyrenoline can be characterized by the specific violet color it forms on the plate (identification limit = 0.01 μg).

The various methods show promise for use in air pollution studies.

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THE QUANTITATIVE SEPARATION OF TETRAPHENYLPORPHINES BY THIN-LAYER CHROMATOGRAPHY

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Previous work demonstrated that tetraphenylporphines (TPP) are formed in the course of ultraviolet irradiation of mixtures of benzaldehyde, pyrrole and water[1,2]. The isolation of TPP from other pyrrole containing compounds formed was effected utilizing a modification of the column chromatographic method of PRIESTHOFF AND BANKS[3].

The need arose for a method of isolation of TPP which was less time consuming, more sensitive, and more reproducible.

Thin-layer chromatography has been used successfully for the separation of the naturally occurring coproporphyrins I and III[4] and of various porphyrin esters[5].

The method described herein represents the application of thin-layer chromatography to the rapid, quantitative isolation of TPP from a complex mixture of pyrrole-containing compounds.

EXPERIMENTAL

All chromatographic plates used in this method were of ordinary type of glass measuring 20.0 × 5.0 cm. Camag Kiesel Gel DO adsorbent is spread in uniform layers of 20 μ thickness from a slurry of 30 g of gel in 70 ml of 50 % ethanol using the Shandon Unoplan spreader. After spreading, the plates are air dried for approximately one-half hour, then activated for at least 1 h at 115°. After cooling, 25 μl of the sample are applied at about 1.5 cm from the bottom of each plate using a 50 μl or 100 μl microsyringe. The plates are developed in a Shandon T.L.C. Chromatank charged with 1 % acetone in benzene (v/v) for a period of about 2 h, *i.e.* until the front travels at least 18.5 cm. The atmosphere in the tank is equilibrated with the developing solvent for at least 1 h before the plates are inserted. After the development of the plates, they are removed from the tank and air dried. The tetraphenylporphine band is located by illuminating the plate with 2537 Å mercury line light (Mineralight). The silica containing the band is scraped off with a microspatula and placed in the stem of a 9 cm long stem funnel, plugged with cotton at the bottom. The elution of this column is accomplished with pure benzene until 3 ml of effluent are collected.

The spectra of the eluted substance are recorded on the Bausch and Lomb, Spectronic 505 recording spectrophotometer in the range from 350 to 650 mμ. Bands at 418 mμ (Soret band) or 515 mμ are used for quantitative determination of the tetraphenylporphine. For quantitative estimation at the 515 mμ maximum, a molar

extinction coefficient of $18.7 \cdot 10^3$ and at the Soret band, *i.e.* 418 mμ, one of $478 \cdot 10^3$ for the free base were used as suggested by THOMAS AND MARTELL[6].

The standard tetraphenylporphine was prepared by the method of BALL, DOROUGH AND CALVIN[7] and its spectrum agreed with those published in the literature[6]

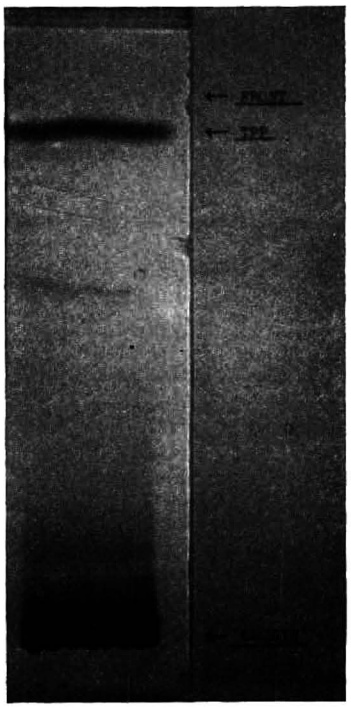

Fig. 1. The thin-layer chromatographic separation of TPP.

RESULTS

Having found the solvent system which would separate TPP from all other compounds of our experimental solutions (Fig. 1) we tested those factors inherent in the overall method which were potential sources of error in the reproducibility and/or accuracy of the determinations.

In order to evaluate the reproducibility of measurement of 25 μl of the solution using a 100 μl microsyringe, 18 samples of standard TPP solution were measured and dissolved directly in 3.0 ml of benzene. The average of the absorbancies of these solutions at 515 mμ was found to be 0.240 with a standard deviation of 0.013 units. Upon calculation it was found that each solution contained 23.7 μg of TPP (Table I-A).

In all subsequent experiments wherein the efficiency of recovery is in question the recovery of the experimental, chromatographed sample is compared with a "standard" sample, *i.e.*, the same volume of TPP solution dissolved directly in 3.0 ml of benzene.

The quantitative recovery of standard TPP was found to be dependent on the method of elution of TPP from Kiesel Gel DO (Table I-B). Fifteen samples of standard

TABLE I

THE RECOVERY OF TPP UNDER VARIOUS CONDITIONS

(See text for description of sample treatments A, B, C, D.)

No. of samples	*Absorbancies ± S.D. (μg)*		*% Recovery*
	515 mμ	*481 mμ*	
A 18 (std TPP)	0.240 ± 0.013 (23.7 μg)		
B 17 (std)	0.237 ± 0.002 (23.4 μg)		
B 15 (batch elution)	0.213 ± 0.014 (21.0 μg)		90%
B 12 (std)	0.275 ± 0.005 (27.1 μg)		
B 14 (column elution)	0.262 ± 0.006 (25.8 μg)		95%
B 15 (std)		0.335 ± 0.012	
B 14 (column elution)		0.319 ± 0.005 (0.14 μg)	95%
C 5 (std)		1.012	
C 5 (irrad)		0.281	
		1.293	
C 5 (mixture A)		1.203	93.5
C 5 (std)		0.925	
C 5 (irrad)		0.303	
		1.228	
C 5 (mixture B)		1.171	95.5
C 5 (std)		1.006	
C 5 (irrad)		0.298	
		1.304	
C 5 (mixture C)		1.227	94.5
D 15 (o t me)		0.303	
D 5 (+ 20 min)		0.301	
D 5 (+ 3.5 h)		0.307	
D 5 (+ 20 h)		0.300	

TPP spotted on TLC plates were scraped off the plates and placed in 3 ml benzene, shaken and centrifuged. The supernatant produced a spectrum with a band at 515 μ of 0.213 ± 0.014 units of absorbancy. This value was compared with the absorbancy produced by a 25 μl sample dissolved directly in 3 ml of benzene and producing a band of 0.237 ± 0.002 units of absorbancy. A yield of 90 % was obtained using this batch method of elution.

However, if elution was done by microcolumn elution, as described in the experimental procedure, the yield was 95 %. The absorbancies obtained on 14 chromatographed samples was 0.262 ± 0.006 and by direct dissolution of the same amount in benzene, the absorbancy was 0.275 ± 0.005 for 12 samples. The total amount of TPP applied in all cases was 27.1 μg. Lowering the total amount of TPP to 0.14 μg still yields 95 % recovery. Fourteen chromatographed samples produced the Soret Band with absorbancy of 0.319 ± 0.005, as compared with an 0.355 ± 0.012 absorbancy obtained on samples dissolved directly in 3 ml of benzene.

The recovery was further checked by mixing the standard TPP with irradiated mixtures of benzaldehyde and pyrrole and then chromatographing, separately, each component and the mixtures. The average absorbancies, at 418 mμ, of these different

standards and mixtures was obtained on three different days and are presented in Table I-C.

The effect of the length of air drying of the developed TLC plates was checked by chromatographing 15 plates containing the same amount of standard TPP solution. The material eluted immediately after development of the chromatograms yielded an average absorbancy of 0.303 at the Soret band (418 mμ). Five plates eluted after 20 min of drying produced a band at 418 mμ with an average absorbancy of 0.301; five plates eluted after $3^1/_2$ h produced an average absorbancy of 0.307 and five plates eluted after 20 h produced an average absorbancy of 0.300 units. It has been concluded therefore, that the time of drying after development has no measurable effect on the recovery of TPP (Table I-D).

The examination of other *i.e.* lower portions of TLC plates did not reveal any Soret band producing compounds. Therefore, it might safely be assumed that the separation of TPP is quantitative.

ACKNOWLEDGEMENTS

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SUMMARY

A method for the isolation and quantitative recovery of α, β, γ, δ-tetraphenylporphine using thin-layer silica gel chromatography is presented. Possible sources of error in the method were tested and found to be negligible.

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AN IMPROVED METHOD FOR THE DETERMINATION OF AMINO ACIDS BY SPECTRAL REFLECTANCE

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INTRODUCTION

The promise of such advantages as reduced tailing, increased sensitivity, and greater speed and resolution[1] has aroused considerable interest in the application of thin-layer chromatography to the analysis of amino acids. In an effort to enhance the utility of the technique for this purpose, the authors have devised a procedure for the *in situ* determination of amino acids resolved on chromatoplates by means of spectral reflectance[2]. The precision attained with this procedure was found to be limited by elements associated with the color development process, such as the incomplete reaction of the acids with the ninhydrin reagent and the leaching out of the acids during the spraying operation.

Since the degree of precision thus achieved was less than that provided when the same procedure without the chromogenic step was applied to a stable system[3], it was felt that substantial improvement in the method would result if the color were developed without sprays. Accordingly it was decided to investigate the possibility of adapting a non spray method, suggested by EL KHADEM *et al.*[4] for the identification of amino acids and sugars separated on paper chromatograms, to the problem at hand. By adding the detecting reagents to the solvent mixtures, these investigators succeeded in eliminating not only the spraying operation but also the drying step preceding it.

EXPERIMENTAL

The amino acids used for this study (DL-alanine, L-arginine, L-glutamic acid, glycine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, DL-serine and DL-valine) were of Calbiochem A Grade purity. Stock solutions of the acids containing 500 mg in enough distilled water to make 50 ml of solution were applied as spots by means of a Hamilton microsyringe in 5 μl increments. The 20 $\times$ 5 $\times$ 0.35 cm plates used for one-dimensional analysis as well as the 20 $\times$ 20 $\times$ 0.35 cm plates employed for the two-dimensional resolutions were coated with Merck silica gel G according to the procedure given in an earlier paper[2]. After resolutions had been achieved, the plates were heated in a mechanical convection oven at 60° for 30 min to dry them and to develop the colors.

Both one- and two-dimensional chromatograms were used in investigating the applicability of four solvent mixtures: (1) *n*-propyl alcohol–water–acetic acid (64:36:20); (2) *n*-butyl alcohol–water–acetic acid (60:20:20); (3) phenol–water (75:25); and (4) *n*-propyl alcohol–34% ammonia (67:33). The first three solvent systems were

employed in conjunction with one-dimensional analyses carried out by the ascending technique. Systems three and two, and four and one were paired off during the two-dimensional analyses with the first of each pair being used for the initial development. Chromatograms were dried at 60° for 30 min prior to development in the second dimension. Successful resolutions of mixtures of the ten amino acids were achieved in 10 h or less during which the solvent fronts were permitted to move 18 cm in each dimension by the ascending technique.

The acids were identified by using R_F values[1] or, in ambiguous situations, by simultaneously running standards for comparison purposes. A Beckman Model DU Spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed for the quantitative evaluation of the spots, which were scraped off the chromatographic plates and worked up into 40 mg analytical samples. The cells used to hold the samples and reference material as well as the procedure followed in preparing material for examination have been described elsewhere by the authors[2,3].

RESULTS AND DISCUSSION

Identification of amino acids

When solvent mixtures containing 0.2 % ninhydrin were used to develop one-dimensional chromatograms, it was not possible to observe the coloration noted by EL KHADEM *et al.*[4] with paper chromatograms when the solvent front reached the amino acids. The spots that appeared after drying, however, were sharply defined and exhibited no tailing. An increase in the ninhydrin concentration to 0.4 % did succeed in producing a faint pink color during the development of the plates, which made it

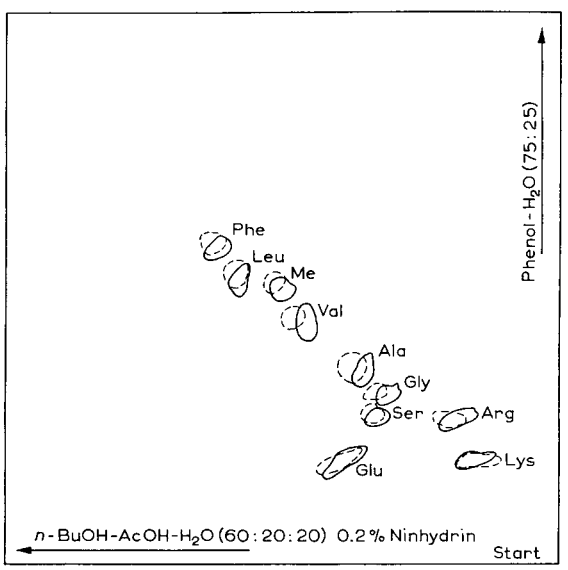

Fig. 1. Two typical chromatograms obtained by using solvent mixture three for the initial development and 0.2 % ninhydrin in solvent mixture two for the second development. Trial one, dotted line; trial two, solid line.

possible to follow the movement of the spots, as well as a greater sensitivity. This last was offset somewhat by the appearance of some tailing.

A clean separation of a mixture consisting of 3 μg of each of the ten amino acids was achieved in 8 to 10 h when solvent three without ninhydrin was used for the initial development and solvent two which was 0.2 % with respect to ninhydrin was employed in the second dimension. Some idea of the degree of resolution attained as well as the feasibility of using R_F values for the identification of the acids can be gained from a consideration of Fig. 1, which shows one chromatogram obtained by means of this procedure superimposed on a second. It should be remembered that the thickness of the adsorbent layer was somewhat greater than usual so as to provide enough material for the determination of the acids, and that the adsorbent was applied not with a precision applicator but manually with masking tape and a glass rod.

Similar success, insofar as resolution is concerned, was realized when solvent mixture four was employed for development in the first dimension and 0.2 % ninhydrin in solvent mixture one for development in the second. As may be seen in Fig. 2,

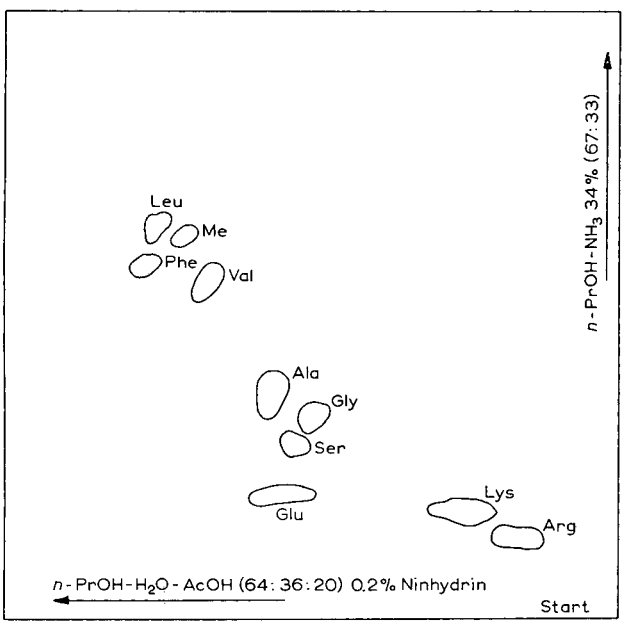

Fig. 2. Typical chromatogram obtained by using solvent mixture four for the initial development and 0.2 % ninhydrin in solvent mixture one for the second development.

which depicts a typical chromatogram obtained with these solvent systems, it is possible to make use of ammoniacal solvents in these separations provided that they are employed for the first development only and provided that the ammonia is removed by a drying operation preceding the second development. Enough adsorbed ammonia remains, however, to impart a brownish or purplish tinge to the entire plate. This results in a lack of contrast between the color of the adsorbent and that of the ninhydrin complexes of the amino acids which renders ammoniacal solvents unsuitable for use in conjunction with the method described herein.

Determination of amino acids

The reproducibility that can be expected of the method was determined by chromatographing four 5 μg replicates of each acid over a distance of 15 cm in one dimension by the ascending technique and preparing them for analysis according to the procedure outlined in the experimental section. When solvent mixture one to which 0.2 % ninhydrin had been added was employed to develop the plates, an average standard deviation of 0.49 % *R* was obtained for the ten sets. As may be seen in Table I, which

TABLE I

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE NO. 1 (0.2 % NINHYDRIN)

Amino acid	*Range* (% *R*)	*Mean* (% *R*)	*Std. dev.* (% *R*)
Alanine	72.8–73.6	73.0	0.39
Arginine·HCl	80.3–81.6	80.8	0.59
Glutamic acid	80.5–81.7	81.2	0.51
Glycine	77.3–77.9	77.5	0.27
Leucine	73.8–74.5	74.2	0.30
Lysine·HCl	79.0–80.9	79.8	0.84
Methionine	78.3–79.4	78.8	0.58
Phenylalanine	81.3–82.6	82.3	0.71
Serine	75.5–76.4	76.1	0.41
Valine	74.2–74.7	74.4	0.27
		Av. std. dev.	0.49

summarizes the results of this experiment, the largest standard deviation found for any one set was the 0.84 % *R* value observed with lysine. Similar data were obtained using solvent mixture two which was 0.2 % with respect to ninhydrin.

These results represent a considerable increase in precision over that provided by spray methods. An average standard deviation of 1.45 % *R* and a maximum standard deviation for a single set of 2.32 % *R* were found for a previous study[2] conducted with three 30 μg replicates of the same ten acids. The two studies differed principally in the solvent systems used and in that the ninhydrin was applied as a spray. Since the results of this research indicate that precision changes of the magnitude being discussed were not observed when the solvent systems were varied, one must ascribe the increase in reproducibility to the elimination of the spraying operation. This conclusion is in accord with the findings reported by JELLINEK AND FRIDMAN[5] who carried out a critical evaluation of the errors in a densitometric analysis of glycine on paper chromatograms.

The effect produced by increasing the ninhydrin concentration was ascertained by repeating the preceding experiment with solvent three to which 0.4 % ninhydrin had been added. As indicated in Table II, the reproducibility attained was only slightly less than that observed with 0.2 % ninhydrin solutions. An average standard deviation of 0.53 % *R* was obtained for the ten sets; the largest standard deviation found for any one set was 0.87 % *R*. There was, however, an increase in sensitivity from the 1.0 μg value found for most of the acids with solvents one and two that had

TABLE II

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE NO. 3 (0.4 % NINHYDRIN)

Amino acid	*Range* (% *R*)	*Mean* (% *R*)	*Std. dev.* (% *R*)
Alanine	50.2–51.1	50.8	0.60
Arginine·HCl	58.3–59.8	59.0	0.66
Glutamic acid	57.7–58.9	58.3	0.57
Glycine	55.8–56.9	56.3	0.56
Leucine	51.0–51.6	51.2	0.30
Lysine·HCl	56.3–57.6	56.9	0.61
Methionine	55.9–57.0	56.7	0.57
Phenylalanine	59.0–60.9	60.1	0.87
Serine	54.2–54.8	54.4	0.30
Valine	52.7–53.5	53.2	0.34
		Av. std. dev.	0.53

been made 0.2 % with respect to ninhydrin to the 0.5 μg value observed with the 0.4 % ninhydrin solution of solvent three. These last results are essentially the same as those obtained when the ninhydrin was applied as a spray[2].

As expected there was some decrease in reproducibility where the ten acids were chromatographed in two dimensions, though the precision was still considerably better than that achieved in one dimension with the use of sprays. The results obtained when four 5 μg replicates were chromatographed in the first dimension with solvent mixture three and in the second dimension with solvent mixture two to which 0.2 % ninhydrin had been added are presented in Table III. The average standard deviation for these data was 0.77 % *R*, with no standard deviation in excess of 1.18 % *R* being found for any of the acids.

TABLE III

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN THE FIRST DIMENSION WITH SOLVENT MIXTURE NO. 3 AND IN THE SECOND DIMENSION WITH SOLVENT MIXTURE NO. 2 (0.2 % NINHYDRIN)

Amino acid	*Range* (% *R*)	*Mean* (% *R*)	*Std. dev.* (% *R*)
Alanine	67.2–68.1	67.8	0.41
Arginine·HCl	78.3–80.4	79.3	0.90
Glutamic acid	78.7–80.3	79.5	0.78
Glycine	74.3–75.3	74.8	0.41
Leucine	68.8–70.8	69.8	0.74
Lysine·HCl	77.9–80.4	79.3	1.15
Methionine	75.8–77.2	76.6	0.65
Phenylalanine	80.3–82.4	81.1	0.90
Serine	70.6–73.0	72.3	1.18
Valine	70.2–71.5	70.9	0.60
		Av. std. dev.	0.77

Despite the increased reproducibility resulting from the elimination of the spraying operation, elements associated with the ninhydrin reaction continued to be the chief factors limiting the precision of the method. Among the most important of these was the close dependence of the color stability of the ninhydrin complexes of the adsorbed amino acids upon the nature of the solvent system used to develop the chromatoplates and on the temperature at which the developed plates were kept[6]. The effect of temperature was such that developed plates which were stored any length of time had to be maintained below 10° to reduce color density changes to a level consistent with precision requirements of the order discussed above. Under these temperature conditions the solvent mixture containing phenol was found to be preferable to those containing acetic acid from the standpoint of color stability. Over a 24h period no variation in excess of 1 % R was observed in the case of plates developed with solvent mixture three while plates developed with mixtures one and two exhibited variations as large as 3 % R. This increased stability associated with the phenolic solvent was apparent even when it was employed as the first solvent in the development of a two-dimensional chromatogram. To insure maximum precision, therefore, it is essential that plates on which unknowns and reference standards are being resolved be processed at the same time and under identical conditions. By proceeding in this manner, it is possible to cancel out not only variations related to the ninhydrin reaction but also those which occur during the drying of the chromatograms because of oxidation and volatilization of the amino acids.

Errors associated with the other operations constituting the procedure were of secondary importance. Variations attributable to the packing of the sample cell were found to amount to an average standard deviation of 0.2 % R for a stable system whose analysis involved no chromogenesis[3]. Although excessive tailing and poor resolutions can curtail the accuracy considerably, especially if large concentrations of acids are involved, such errors can be avoided to a large degree by choosing suitable separation procedures; by increasing the thickness of the adsorbent layer; and by extending the development time. When working with more than 20 to 30 μg of acid, it was necessary to make the analytical sample larger than 40 mg to accommodate the increased amount of test material as the areas of the spots were approximately proportional to the concentration. To determine the loss of precision that might be attributed to tailing, plates on which were paired developed and undeveloped spots consisting of 30 μg of the same acid were sprayed, dried at approximately 55°, and subjected to analysis by spectral reflectance. In the case of no acid were differences greater than those ascribable to the ninhydrin reaction found between paired spots. This observation is in keeping with results reported for paper chromatography[7,8].

The effect upon reproducibility of varying the slit width of the spectrophoto-

TABLE IV

REPRODUCIBILITY OF REFLECTANCE MEASUREMENTS AS A FUNCTION OF SLIT WIDTH. READINGS OBTAINED AT 515 mμ WITH A SAMPLE OF GLYCINE COMPLEXED WITH NINHYDRIN

Slit width (mm)	0.25	0.3	0.4	0.5	0.6	0.7
Band width (mμ)	5	6	8	10	12	14
Mean of four readings (% R)	67.5	67.5	67.6	67.7	67.5	69.1
Standard deviation (% R)	0.05	0.00	0.05	0.08	0.14	0.22

meter was ascertained by measuring the reflectance at 515 mμ of a 10 μg sample of glycine complexed with ninhydrin relative to a standard consisting of adsorbent removed from the same plate. Four consecutive measurements were made at each of several slit openings using the blue-sensitive phototube at load resistor setting 2 and at sensitivity setting 3. A consideration of the results obtained, which are set forth in Table IV, revealed that standard deviations of 0.05 % R or less can be expected for slit widths in the range 0.25–0.4 mm. There is a decrease in precision with widths greater than this while smaller widths are not suited for the measurement of diffuse reflectance. The band widths isolated at the various slit settings were obtained from dispersion data provided for the Beckman DU spectrophotometer.

Finally the probable relative error in the measurement of the concentrations of alanine, leucine, serine and valine was determined by making use of the precision data obtained with four 5 μg replicates of the acids and listed in Table I, and of the calibration curves for these same acids which were presented by the authors in an earlier paper[2]. A similar investigation was carried out to ascertain the relationship

TABLE V

PROBABLE RELATIVE ERROR IN THE MEASUREMENT OF THE CONCENTRATIONS OF SOME AMINO ACIDS

	Alanine	*Leucine*	*Serine*	*Valine*
Range (% R)	72.8–73.6	73.8–74.5	75.5–76.4	74.2–74.7
Mean (% R)	73.0	74.2	76.1	74.4
Standard deviation (% R)	0.39	0.30	0.41	0.27
Equivalent change in measured concentration of acid (μg)	0.18	0.15	0.25	0.14
Probable % relative error	3.6	3.8	5.0	2.8

between the probable relative error and the concentration of glycine. In this instance solvent one which was 0.2 % with respect to ninhydrin was employed in conjunction with four replicates of acid at each concentration investigated. Data relative to these two studies are presented in Tables V and VI, respectively. The change in measured concentration equivalent to the deviations observed for the various acids was obtained from the appropriate calibration curves and expressed as a probable % relative error in concentration. For the five acids at 5 μg concentration this figure ranged from

TABLE VI

PROBABLE RELATIVE ERROR IN THE MEASUREMENT OF THE CONCENTRATION OF GLYCINE AS A FUNCTION OF CONCENTRATION

	Concentration of glycine (μg per spot)			
	2	5	10	20
Range (% R)	84.3–86.0	77.3–77.9	63.6–65.1	55.3–57.1
Mean (% R)	85.1	77.5	64.4	56.3
Standard deviation (% R)	0.92	0.27	0.66	0.76
Equivalent change in measured concentration of glycine (μg)	0.18	0.20	0.50	1.40
Probable % relative error	9.0	4.0	5.0	7.0

a low value of 2.8 % for valine to a high of 5.0 % for serine. In the case of glycine minimal values were obtained in the intermediate concentration range. The relatively large 9.0 % value observed at the 2 μg concentration may be attributed to the fact that this concentration approached the 1 μg sensitivity limit for glycine as well as to the increased contribution of volumetric and gravimetric errors associated with such operations as the preparation of the standard solutions. At the opposite end of the scale, the 7.0 % figure found for 20 μg concentrations can be ascribed to the flattening of the calibration curve that occurs at high concentrations. Of the acids investigated, this effect is particularly noticeable in the case of glycine[2].

CONCLUSIONS

The application of a nonspray method devised by EL KHADEM *et al.*[4] for use in paper chromatography to the determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates has enhanced the utility of the latter technique considerably. By dissolving ninhydrin in the solvent mixtures employed to develop the chromatograms, one drying and the subsequent spraying of the plates are eliminated. Despite this modification, the R_F values of the amino acids are not altered with the result that values obtained by means of the conventional spray method can still be utilized for purposes of identification. The nonspray procedure can be applied successfully to two-dimensional chromatography by adding the ninhydrin to the second solvent system. When no quantitative work is contemplated, ammoniacal solvents can be employed for the first development provided that the ammonia is removed by drying prior to the development in the second direction.

Depending on the concentration of ninhydrin used, the sensitivity of the method is between $5 \cdot 10^{-9}$ and $1 \cdot 10^{-8}$ moles for most amino acids[2]. Although this is somewhat less than that afforded by the *in situ* estimation of amino acids separated on paper, this slight decrease in sensitivity is more than compensated for by an increase in precision and accuracy. The elimination of the spraying operation with its attendant irregularities, such as leaching out of the amino acids, has resulted in a precision which is comparable to that reported for reflectance measurements made of spots on paper, and which is better than that reported for transmission measurements made of these same spots through the paper[9]. The comparison becomes all the more favorable when one considers that the latter data were obtained with a stable system —copper rubeanate—and with undeveloped spots. The overall accuracy attained in the measurement of the concentrations of amino acids equals or exceeds that achieved when such techniques as total scanning and the electronic integration of curves are employed in conjunction with paper chromatograms[5,10,11].

Although the present study was restricted to amino acids, there appears to be no reason why an appropriate modification of the procedure described herein cannot be applied to other systems. For example, EL KHADEM *et al.*[4] have also employed the nonspray technique for the separation of sugars on paper while BEVENUE AND WILLIAMS[12] have devised a method for the direct estimation by reflectance spectrometry of sugars separated by paper chromatography. It should be possible to make use of these results in conjunction with such procedures for the resolution of sugars on thin-layer plates as those proposed by STAHL AND KALTENBACH[13] and by PASTUSKA[14].

SUMMARY

A nonspray method is described whereby amino acids resolved on thin-layer plates can be estimated by spectral reflectance. In addition to facilitating the determination the elimination of the spraying operation with its attendant irregularities has resulted in a substantial increase in precision and accuracy. In this respect the method is competitive with paper chromatography, used in conjunction with such techniques as total scanning and the electronic integration of curves.

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THE AMINO ACID COMPOSITION OF HEMOGLOBIN

IV. THE PREPARATION OF PURE POLYPEPTIDE CHAINS OF HUMAN HEMOGLOBINS*

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Pure polypeptide preparations are vital to the successful amino acid analysis of any protein material. For those dealing with the analysis of hemoglobin, large scale separation of the protein into its component polypeptide chains has been carried out by means of column chromatography on Amberlite IRC-50 utilizing a urea gradient at pH 1.9[1,2], by elution from carboxymethyl cellulose (CMC) with pyridine buffers[3], by fractional precipitation with trichloroacetic acid (TCA)[4,5] and by countercurrent distribution[6]. Each of these methods presents serious difficulties or shortcomings. The IRC-50 technique, though easiest to do, fails to provide clean non-α-chains in the case of human hemoglobin, although essentially pure preparations of the α-chains are easily obtained. The CMC technique is highly dependent on the individual batch of cellulose employed, is thus difficult to standardize, and, in our hands, has not given sufficiently clean preparations of the peptide chains. At first glance, the fractional precipitation method of HAYASHI[4] would seem to provide ease and reliability for such separations, but we have never been able to achieve pure non-α-chain preparations while on only rare occasions have we been able to prepare relatively clean α-chain material using this technique. The countercurrent method requires equipment not available in many laboratories as well as very careful attention to details. Furthermore, clean α-chain preparations are not uniformly obtained by this procedure. In view of these difficulties, we have attempted to incorporate the better aspects of several of these techniques into a method which would provide pure α- and non-α-chain preparations of human hemoglobins, would be simple to perform, easy to standardize and would utilize unspecialized equipment readily available in most laboratories.

METHODS AND MATERIALS

Hemoglobin solutions were prepared by standard techniques of washing the erythrocytes with saline and lysing by water and toluene or water and carbon tetrachloride. Hemoglobin preparations were purified by chromatography on CMC[7] or DEAE cellulose[8]. Frequently, rechromatography on one or the other ion exchanger or successive passage through both kinds of cellulose was carried out until the preparations were judged to be homogeneous by starch gel electrophoresis.

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Globin preparations were obtained from the pure hemoglobin fractions by precipitation with acid acetone in a scaled up version of a technique previously described[9], which is based on the method of ANSON AND MIRSKY[10].

Chromatography on IRC-50

Amberlite columns, prepared essentially as previously described[2], were utilized for the initial phase of the separation of the α- from the non-α-chains. The resin, CG-2, was purified as described by HIRS, MOORE AND STEIN[11] and sized by the hydraulic technique of HAMILTON[12] to obtain a more uniform particle range and thus improve the flow rates. Columns, 1.9 × 65 to 70 cm, resin height, were poured in one successive batch by permitting a slurry of Amberlite to enter the top of the column as the suspending buffer flowed slowly from the bottom. Columns poured in this fashion have flow rates of from 100 to 150 ml/h at room temperature. The clean, sized resin in its acid phase was equilibrated with 11.7 % formic acid before pouring, and the completed column was further equilibrated with 500–1000 ml of this same material. From 500 to 800 mg globin were dissolved in 11.7 % formic acid at approximately a 1 % concentration and the clear solution added directly to the top of the column. 500 ml of 2 *M* urea, brought to pH 1.9 with concentrated HCl, were allowed to pass through the column slowly overnight, during which time non-heme proteins and non-protein materials are eluted. The elution buffer was then changed to 5 *M* urea, pH 1.9, and the effluent monitored at 280 mμ utilizing a Gilson Medical Electronics instrument. Fraction I consists of the effluent containing α-chain material which was collected from the time of the initial rise of the absorption at 280 mμ until the recording graph demonstrated a plateau effect on the descending limb of this peak (Fig. 1). Approximately 300–400 ml of 5 *M* urea suffice to recover the α-chains, following which 8 *M* urea, pH 1.9 was utilized for the elution of the remaining material. Fraction II consists of a mixture of approximately equal amounts of α- and β-

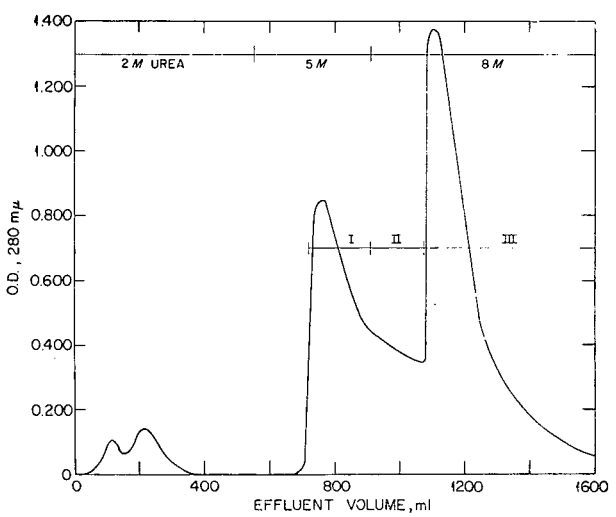


Fig. 1. Chromatography of globin from Hgb A into α- and non-α-polypeptide chains. Amberlite IRC-50, pH 1.9; column dimensions: 1.9 × 65 cm; 500 mg globin A; elution buffers: 2 *M*, 5 *M* and 8 *M* urea, pH 1.9; optical densities at 280 mμ. See text for definition of fractions I, II and III.

chains and represents the material collected from the time the elution buffer was changed from 5 *M* to 8 *M* urea until the sharp rise in optical density signals the elution of the β-chains. Fraction III consists of α- and β-chains in a ratio of approximately 1:3 and comprises the remaining effluent from the column. From 600 to 800 ml of the 8 *M* urea are sufficient to clear the column of α- and β-chains.

Fraction I

This material contains 95–98 % α-chains and may be dialyzed *versus* repeated changes of distilled water in the cold until free of urea (14 to 18 changes of the dialysis fluid will be required). The dialyzed material is then freeze dried. A yield of 35 % of the starting material is usually obtained. An alternative method of preparation eliminates the long dialysis procedure mentioned above and depends on the precipitation of the protein by TCA followed by washing with acetone to remove excess reagents. After reducing the urea content with 2 to 4 changes of the dialysis fluid, the polypeptide is precipitated by the addition of sufficient TCA to constitute an approximately 0.6 *M* solution (10 g per 100 ml of solution). The resulting precipitate is washed 3 times by centrifugation with acetone and dried under vacuum. This procedure yields a product indistinguishable in behavior and amount from that obtained by the method described above.

Fractions II and III

These fractions must be repurified to obtain satisfactory β-chain preparations. Dialysis to remove much of the urea is carried out against 2 to 4 changes of distilled water and the peptide mixtures are precipitated by the TCA technique described above. The dried samples, representing approximately 15 % of the starting material in fraction II and 40 % in fraction III are subjected to a modified countercurrent separation according to the technique of BOWMAN AND INGRAM[13]. 200 ml of 1 % aqueous dichloroacetic acid are mixed several times with 200 ml of reagent grade *sec.*-butanol in a separatory funnel and allowed to equilibrate for 1 h at room temperature. The two phases are drawn off separately. 8 ml of the lower phase are introduced into a series of 18 test tubes, 15 × 125 mm. To tube No. 1 are added up to 125 mg of fraction II or III and the mixture agitated until maximal dissolution is achieved. 8 ml of the upper phase are then added to tube No. 1 and the mixture again shaken, at which time any undissolved material will have been noted to go into solution. The stoppered tube is centrifuged at 2000 r.p.m. for 5 min at 5°, which results in a clean separation of the two phases. The upper phase of tube No. 1 is transferred to tube No. 2 and approximately 8 ml of fresh upper phase are added to tube No. 1. In transferring the upper phase of tube No. 1 to tube No. 2, any whitish suspension should be allowed to remain with tube No. 1 and care should be taken not to transfer any of the lower phase to tube No. 2. The tubes are mixed, centrifuged and transferred as before to each successive tube until the 18th transfer has been achieved. Protein determinations on the well mixed contents of each tube are carried out on a small aliquot by the method of LOWRY *et al.*[14]. A curve of the optical densities at 750 mμ will demonstrate three peaks, the initial two containing variable quantities of α- and β-chains, the third, 93–98 % β-chains (Fig. 2). The latter material is harvested by washing fraction C with ether in a separatory funnel and freeze drying the aqueous layer. Peaks A and B may be repurified by an additional passage through

the 18 tube transfer system. One may thus obtain a yield of approximately 35 % purified β-chains in addition to a like amount of similarly purified α-chains.

Starch gel electrophoresis of the individual fractions was carried out in urea–veronal buffer, pH 8.05, by methods previously described[9].

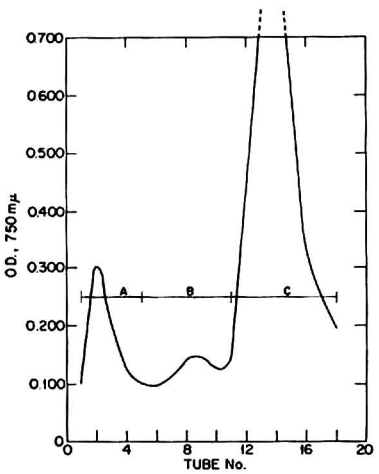


Fig. 2. Separation of non-α- from α-chains of globin A by countercurrent distribution. Protein determinations done on 0.2 ml aliquots from each of the 18 tubes by the method of LOWRY *et al.*[14]. See text for definition of fractions A, B and C.

Peptide mapping of the tryptic digest of the individual purified chains was carried out by chromatography in a butanol–acetic acid–water (4:1:5) system and electrophoresis at pH 3.7 or 6.4 in pyridine–acetic acid–water buffers by techniques previously described[15,16]. A determination of the amino acid composition of the individual peptides or of the purified polypeptides was carried out by hydrolysis with 6 *N* HCl in sealed evacuated tubes for 24 or 72 h at 108° followed by analysis on an automatic amino acid analyzer by the method of MOORE, SPACKMAN AND STEIN[17].

DISCUSSION

Of the various methods described for the preparation of hemoglobin polypeptide chains of sufficient purity for further analysis, the countercurrent distribution technique yields the best material. Even with this technique, however, the α-chain peptides frequently display excessive contamination with non-α-components. Furthermore, highly specialized, expensive equipment is needed to carry out a sufficient number of transfers to promote separation of the two types of chains. Although the Amberlite technique is the easiest with which to deal, sufficiently pure β-chain preparations cannot be obtained. By combining the better characteristics of these two procedures, it is possible to obtain good yields of sufficiently pure polypeptide chains to carry out quantitative amino acid studies. If one adds to this technique the simplification suggested by the method of HAYASHI[4], that is, the precipitation of the chains by TCA and extraction of the excess reagent by acetone or ether, one has a

reliable, relatively uncomplicated procedure which yields highly purified fractions in satisfactory quantities for further study.

It is unfortunate that the TCA method of HAYASHI[4] has not been found to work adequately in our laboratory since it would appear to be ideal for the purposes at hand. Among the reasons for this difficulty may be mentioned the following. Perhaps the most critical problem in using TCA for differential precipitation is the difficulty in preparing precisely similar TCA solutions in view of the hygroscopic nature of the chemical. Absolute control of the temperature of precipitation seems to be needed and the end point of titration is exceedingly difficult to visualize. Even in our best preparations, gross (10–20 %) contamination of one chain with another could be seen on starch gel analysis. Nevertheless, precipitation with TCA does permit one to deal with the purified material obtained in the method described in this manuscript in a more expeditious way.

DINTZIS[3] has pointed out that different batches of CMC yielded different degrees of separation of the polypeptide chains of animal hemoglobins[3]. We have tried four different preparations of CMC, including one home-made batch, without success in achieving a clean-cut separation. The reasons for our lack of success may well reside in the fact that only specific lots of the exchanger seem to work.

Our experience with the technique described in this presentation suggests that it is more than adequate to achieve a satisfactory product. Starch gel patterns of the isolated fractions obtained reveal no visible contamination of one chain by the other (Figs. 3, 4 and 5) although quantitative amino acid analysis suggests the possibility of traces of such contamination, amounting to significantly less than 5 % in most preparations. This degree of contamination is insufficient to show up on peptide mappings as can be seen in Figs. 6 and 7. We have used the method with equal success with hemoglobins possessing abnormal α-, β- or δ-chains. To date we have not had an opportunity to study abnormal γ-chains but we presume they would act in

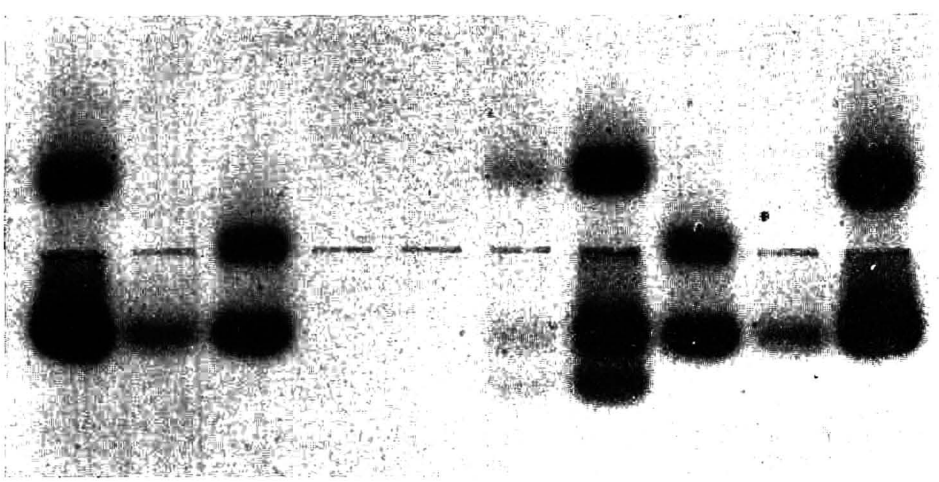


Fig. 3. Starch gel electrophoresis of polypeptide chains of human globin in urea–veronal buffer, pH 8.6[9]. Anode at the top. Slots numbered from left to right. Stained with AB-10. Pure α-chain preparations in slots 2 and 9. Normal globin (α- and β-chains) in slots 1 and 10. Globin from Hgb A_2 (α- and δ-chains) in slots 3 and 8. Globin from a mixture of normal hemoglobin and hemoglobin with abnormal α-chains in slots 6 and 7.

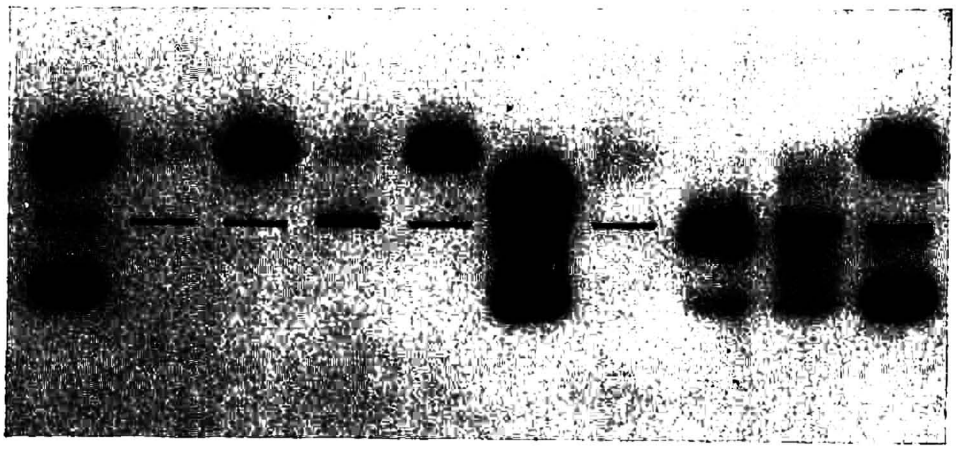


Fig. 4. See legend of Fig. 3. Purified β-chains from fraction C seen in slots 2, 3, 4 and 5.

a comparable fashion. In evaluating this procedure, several aspects are worthy of comment. By pouring the Amberlite column in a continuous slurry, sharper peaks seem to have been achieved. It is also likely that the hydraulically sized resin has contributed to the cleaner separation of the α-chain in comparison to previous work with this system. Certainly, the flow rates, faster than previously observed, are a reflection of the uniformity of the resin particles. Because no gradient is involved in the ion exchange portion of the procedure, the technical set-up of the column has become simpler. We have adopted a urea molarity sufficient to remove most but not all of the α-chains in the resin, but insufficient to start the elution of the non-α-

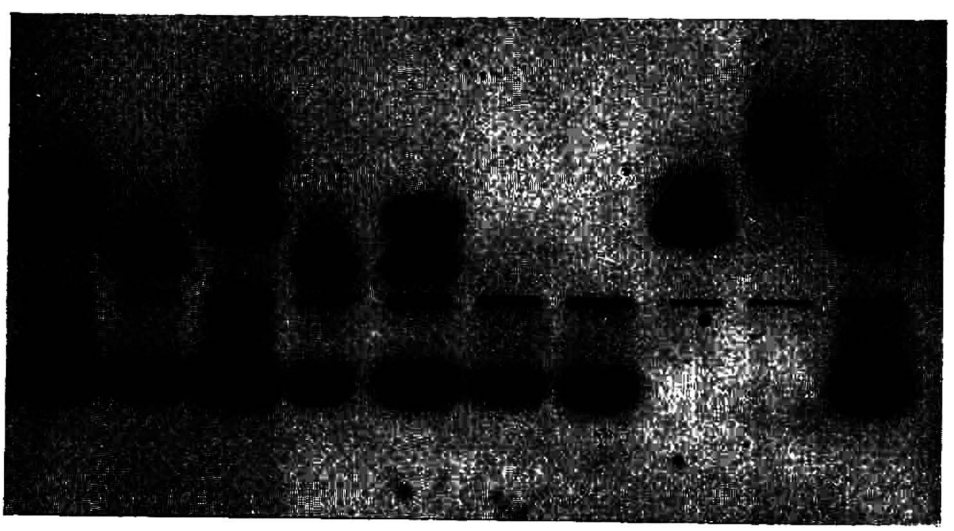


Fig. 5. See legend of Fig. 3. Pure α-chains from Hgb A (from fraction I) in slots 6 and 7. Pure β-chains from Hgb A (from fraction C) in slot 8. Pure β-chains from abnormal Hgb Durham No. 1 (from fraction C) in slot 9. Remaining slots contain globins from a variety of unseparated hemoglobins.

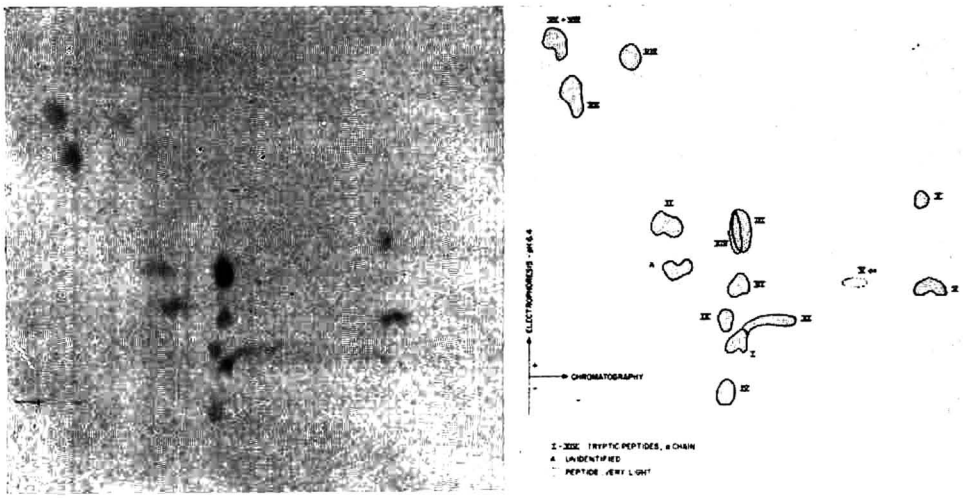


Fig. 6. Tryptic peptide map of α-chain of Hgb A harvested from fraction I of IRC-50 column. 0.5% ninhydrin in 100% ethanol used for stain. The line drawings of the peptides indicate their position and official numbers.

chains. The latter requires 5.3–5.6 *M* urea before the initial traces are eluted. Should one wish to increase the purity of the α-chain preparation, fraction I may be cut off just beyond the point of peaking noted in Fig. 1, with, of course, a corresponding decrease in yield.

Points of note related to the countercurrent distribution aspect of the procedure are as follows. The relatively limited number (18) of transfers employed has proven to be sufficient to provide the degree of purification sought because of several reasons. First, the starting material is already enriched in terms of the non-α-chains and has

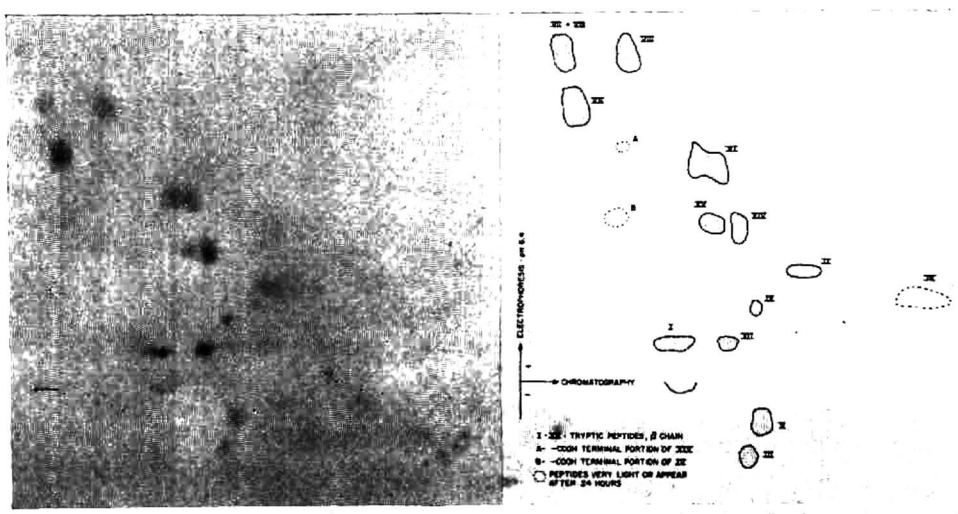
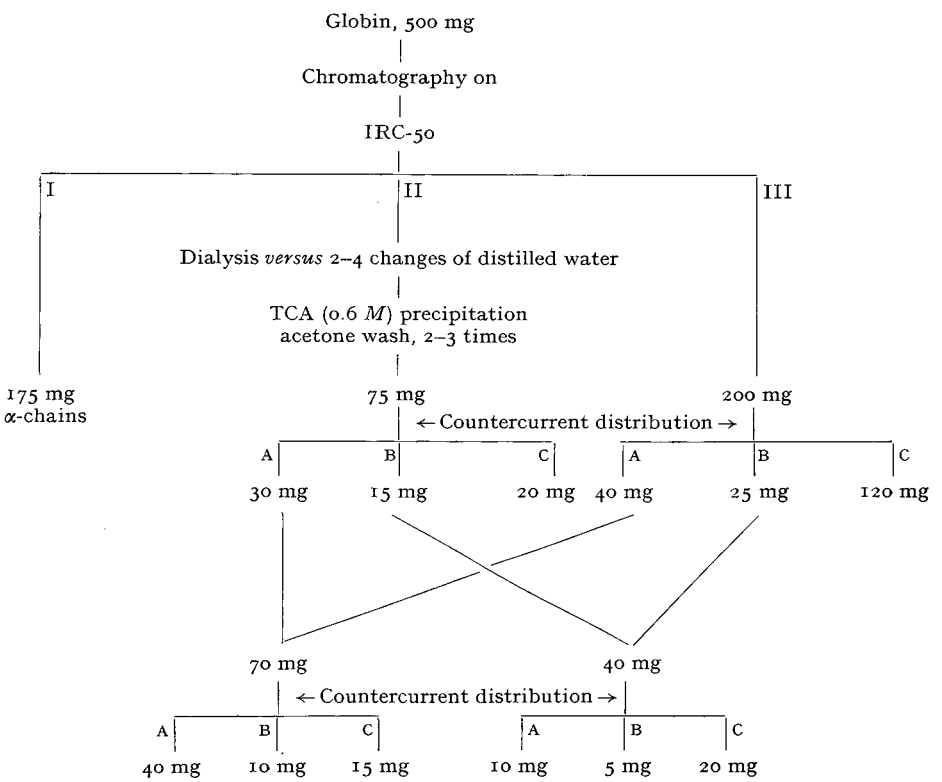


Fig. 7. Tryptic peptide map of β-chain of Hgb A harvested from fraction C of countercurrent distribution technique. Other conditions as in Fig. 6.

relatively little contaminant to separate. Second, by centrifuging the tubes, very clean separation between the two phases is achieved and this separation is weighted in favor of obtaining purer β-chains by leaving a trace of the upper phase undisturbed in each transfer. Such methods cannot, of course, be utilized to determine distribution constants, but for the purposes of separation and purification work admirably. Repurification of the non-α-chains may be achieved by re-cycling through the countercurrent procedure and increased yields are thus possible. The method will easily accommodate larger samples by upgrading the volumes used. Finally, it should be noted that a single individual can complete the entire procedure of countercurrent distribution in one working day.

A flow pattern with representative recoveries is outlined below.

SCHEME I



Combined C total = β-chains, 175 mg

SUMMARY

A technique for the preparation of polypeptide chains of human hemoglobins of a high degree of purity is described. The method involves the combined use of ion exchange chromatography and countercurrent distribution.

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THE SYNTHESIS AND CHROMATOGRAPHIC PROPERTIES OF CARBOXYL CELLULOSE*

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INTRODUCTION

The chromatographic separation of compounds with similar chemical characteristics has been one of the important advances in biochemistry, however, these techniques utilizing ion exchange resins were not universally successful in affecting separation of protein mixtures. Ion exchange resins usually form strong bonds with proteins making it difficult to adsorb and desorb the molecules without destroying the comparatively weak bonds which hold them in their native configuration. While the ion exchange resins have a relatively high number of adsorptive sites, the sites are not limited to the surface of the resin particle thus substantially reducing the capacity of the resins for large molecules like proteins.

Cellulose ion exchange materials[1–3] are derived by the controlled substitution of anionic or cationic groups onto the cellulose polymer. The reactions utilized in their synthesis have been known for some time, however, the prime factor in the synthesis is the restriction of substitution to control undesirable changes in physical properties of the cellulose such as formation of a gel or a soluble or granular product. The weak bonds formed between proteins and the ionizing groups on the cellulose may be severed by relatively mild changes of pH or ionic strength. Most cellulose ion exchange materials do not contain as many binding sites per gram as the ion exchange resins, but their charges are confined mostly to the surface making them more available for bond formation. Consequently, the substituted celluloses often bind nearly their own weight in protein.

The variety of binding sites and molecular structures that characterize protein molecules make it reasonable to assume that varying the configuration in which particular groups are placed on cellulose may change the characteristics as an ion exchange material for separation of protein mixtures.

Our purpose was: (1) to investigate the feasibility of converting one or both of the hydroxyl groups on carbon atoms 2 and 3 of the glucose sub-unit in the surface chains of cellulose to carboxyl groups while retaining the basic physical properties of the cellulose; (2) to determine whether the resultant material possessed the properties of a satisfactory ion exchange adsorbent.

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EXPERIMENTAL

Preparation of dialdehyde cellulose

Cellulose with approximately 1 mequiv. carboxyl per gram is produced at room temperature by the initial reaction of 60 g of cellulose (SW-40-B Solkafloc) in 1 l of 0.0435 *M* potassium metaperiodate solution for 16 h[4]. The amount of aldehyde formed can be controlled by limiting exposure of the cellulose to potassium metaperiodate. With excess periodate present, a reaction time of 16 h or less prevents product loss and formation of granular material lacking the ideal flow quality of the natural cellulose. When limiting the reagent to form a specific number of aldehyde groups, 10 % more than the stoichiometric amount must be provided to compensate for the spontaneous decomposition of potassium metaperiodate in aqueous solutions at room temperature. After proper substitution has been achieved the remaining periodate and by-products are removed by repeated suspension of the product in distilled water followed by settling and decantation.

Preparation of carboxyl cellulose

Dialdehyde cellulose (60 g) is added to 2 l of 0.1 *M* acetic acid which is 0.11 *M* with respect to sodium chlorite[5,6]. The reaction is allowed to proceed with continuous mixing for 24 h at room temperature. Because of the noxious fumes generated, the reaction is best carried out in a hood. After oxidation, the supernatant fluid is decanted and the product is washed three times by resuspension in distilled water and filtered through a Buchner funnel. Finally, the product is suspended in 95 % ethanol and filtered. The filter cake is removed, broken into small pieces and allowed to dry in air or under an infrared lamp.

To summarize, each 1.1 mequiv. of potassium metaperiodate will form approximately 2.0 mequiv. of aldehyde which can subsequently be oxidized to 1.5 mequiv. of carboxyl groups. When limiting the reagent, one should provide 0.733 mequiv. of periodate for every mequiv. of carboxyl desired in the final product.

Aldehyde content

Aldehyde content was calculated from data on periodate consumption corrected for spontaneous breakdown under conditions of the reaction. Since each molecule of periodate is capable of producing two aldehyde groups on each glucose sub-

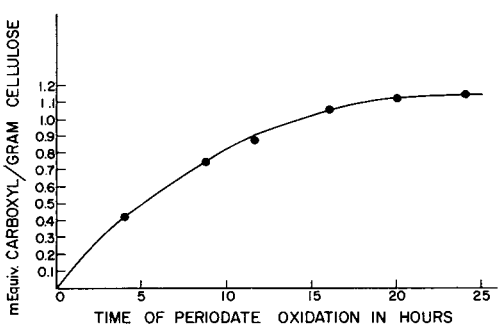


Fig. 1. Increase in titrable carboxyl groups after chlorite oxidation of cellulose samples exposed to periodate from 4–24 h.

unit of the cellulose, the number of mequiv. of aldehyde formed in the reaction is twice the mequiv. of periodate reacting with the cellulose.

The ultimate effect of increased aldehyde content is expressed best by the increase in carboxyl groups produced from cellulose samples treated with periodate for varying periods of time. Fig. 1 shows the increase in titrable acidity when 1 g of cellulose is exposed to 1.5 mequiv. of periodate for periods of 4–24 h with a subsequent 24 h chlorite oxidation. While the rate of carboxyl formation is not linear, it is reproducible when periodate, in excess, is permitted to react with cellulose at room temperature. The most rapid increase in carboxyl content occurs in samples exposed to periodate during the initial 8 h of the reaction. Samples exposed over 16 h tend to decrease in weight due to formation of soluble by-products.

Carboxyl contents

The carboxyl groups in the cellulose were determined by electrometric titration before and after oxidation. The rate at which dialdehyde cellulose is oxidized to carboxyl cellulose is shown in Fig. 2. Fifty percent of the total aldehyde groups present were oxidized within the first hour. This apparently indicates that one of the aldehyde groups is less easily oxidized than the other due to steric hindrance or the negative charge repulsion which would exist at the pH of the reaction. Only 73–75 % of the aldehyde present in the cellulose polymer can be oxidized even when the product was exposed to chlorous acid for several days. A slight decrease in carboxyl content has been observed when chlorous acid oxidation is continued for longer than 24 h.

Electrometric titration

Representative titration curves of carboxyl cellulose are shown in Fig. 3. The pK of 4.55 in water is higher than that reported for carboxymethyl cellulose and most substituted carboxyl groups in aqueous solution. In a 0.2 M sodium chloride solution,

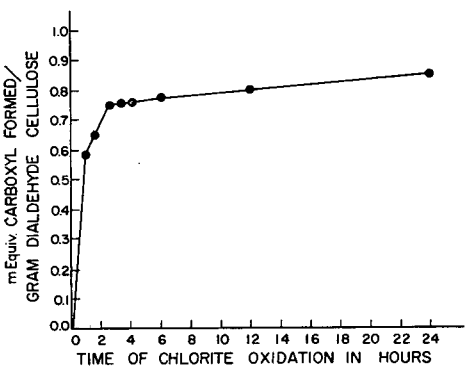


Fig. 2. Increase in titrable carboxyl groups in samples of dialdehyde cellulose exposed to chlorous acid for 0.5–24 h.

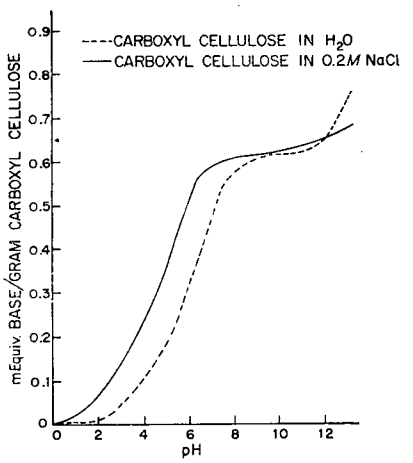


Fig. 3. Titration curves of carboxyl cellulose in distilled water and 0.2 M sodium chloride.

the pK shifts to 3.6, a value close to the one expected. It was not possible to determine from the titration curve whether the titrable groups were homogeneous with respect to pK.

pH stability

There is a measurable reduction of titrable carboxyl groups when carboxyl cellulose stands in solutions below pH 4 or above pH 11 for 24 h. The downward shift in pH noted after the addition of base during the titration of material exposed to pH values below 4 suggests that the loss of titrable groups may have been due to lactone formation. Dialdehyde cellulose was observed to lose weight in basic solutions. It would appear that cleavage of the pyranose ring decreases the stability of the cellulose polymer in alkaline solution.

Ultraviolet absorbing material

Ultraviolet absorbing material is bound on the cellulose after the chlorite oxidation step. This material cannot be completely removed from carboxyl cellulose which is prepared in large batches, consequently, some will appear in the first few ml of eluate. The interfering material can be eluted with about 1.5 bed volumes of the starting buffer.

Separation of serum albumin and lysozyme

Lysozyme is a very basic protein with an isoelectric point of pH 10.7 while the isoelectric point of bovine serum albumin is pH 4.7. Chromatographic separation of a mixture of the two was effected on a 1.2 × 25 cm carboxyl cellulose column using a 1 l linear gradient 0.01–0.50 M citrate buffer pH 5.0. There was no binding of the albumin which emerged in one bed volume while it took 350 ml of buffer to elute the lysozyme. Basic proteins are bound to the ion exchange material and may be eluted under relatively mild conditions.

Chromatography of cytochrome C

An easily obtainable mixture containing basic proteins suitable for chromatography on this ion exchange material is horse heart cytochrome C. Others have reported and we have confirmed the presence of 4 components[7,8] in cytochrome C prepared by the method of KEILIN AND HARTREE.

The chromatography of cytochrome C was performed on a 20 × 1.5 cm carboxyl cellulose column using a 0.2 M sodium phosphate buffer pH 7.04. The fractions observed were: a rapidly moving straw-colored band, reduced and oxidized cytochrome C appearing in a two-component band (the leading edge contained reduced cytochrome C and the remainder oxidized cytochrome C as demonstrated by their respective absorption spectra), and one component which remained at the origin. The straw-colored component may be eluted with distilled water and has been identified by others as myoglobin[7]. The remaining component has resisted all efforts to remove it including elution with 1 N acid or base. The material is thought to be cytochrome C which was denatured during isolation.

Iron determinations were made by the method of THEORELL AND PEDERSEN[9] on 2 mg samples of oxidized cytochrome C which had been dialysed and dried to constant weight. Analysis shows the oxidized cytochrome C to contain 0.43 % iron, a

value identical with that reported by MARGOLIASH[10] for oxidized cytochrome C purified at pH 7.0, but somewhat lower than the 0.46 % iron reported when chromatographic separation was done at pH 9.6.

Effects of pH and ionic strength on elution volume

Elution volume has been defined as the total volume of liquid which flows from the column from time of application of solute until the maximum solute concentration emerges from the column minus the volume of solvent held between the resin particles[8]. These studies were done using 4 g of carboxyl cellulose (0.48 mequiv./g) in a 1.5 cm diameter column, whose height varied with pH and ionic strength. Sodium phosphate buffers of appropriate ionic strength and pH were used to elute 2 mg samples of cytochrome C from the columns. Fig. 4 shows the effect of ionic strength at pH 7.04 on the elution volume of cytochrome C. Elution volumes increase sharply

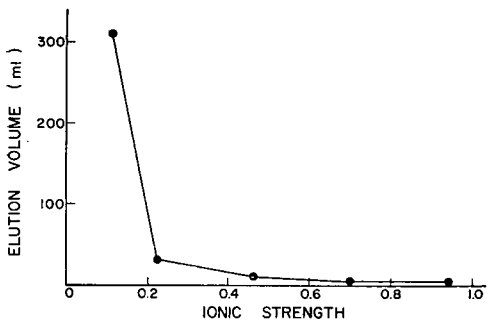


Fig. 4. Effect of ionic strength on the elution of cytochrome C at pH 7.04.

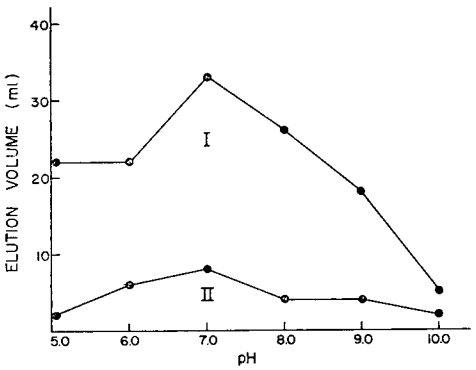
below an ionic strength of 0.2. Cytochrome C appears to remain stationary when the ionic strength is reduced below 0.02. The elution volume is maximal at pH 7.0 with ionic strength 0.22 (I) and 0.46 (II) (Fig. 5) and decreases both above and below this pH. The elution volume of cytochrome C increases below pH 7.0 with the carboxyl-containing resin IRC-50. The reduced elution volume of cytochrome C with carboxyl cellulose at lower pH values may reflect some lactone formation between the carboxyl and adjacent hydroxyl groups.

Adsorption capacity

The capacity of carboxyl cellulose was evaluated with two proteins, lysozyme (approx. mol. wt. 16,000) and cytochrome C (mol. wt. 12,100). At near zero ionic strength and room temperature with a pH of 5.4, 1 g of carboxyl cellulose (0.74 mequiv./g) adsorbs 270 mg of lysozyme and 280 mg of cytochrome C from 200 ml of a 0.1 % solution stirred continuously for 2 h. One gram of carboxyl cellulose (0.48 mequiv./g) adsorbs 183 mg of cytochrome C under similar conditions. It is difficult to compare the capacity of carboxymethyl cellulose with carboxyl cellulose since binding studies were not done with the same proteins. Taking into consideration the number of molecules of protein adsorbed per carboxyl, the capacities appear to be similar. It is considered probable that positions with adjacent carboxyl groups have no greater capacity than similar positions with a single carboxyl present.

Adsorption of lysine

Fig. 6 shows the effect of pH on the capacity of 1 g of carboxyl cellulose (0.48 mequiv./g) to adsorb lysine from 200 ml of a 0.0005 *M* solution. The solutions were adjusted to the specified pH using 6 *N* hydrochloric acid or sodium hydroxide.



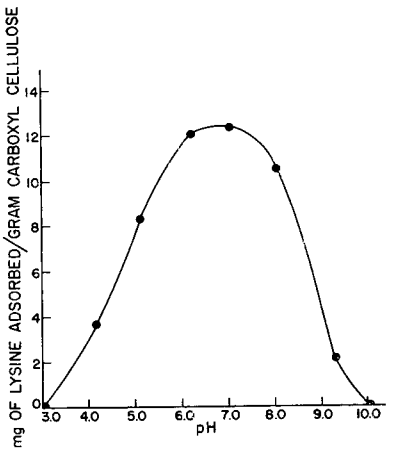


Fig. 5. Effect of pH on the elution of cytochrome C at ionic strength 0.22 (I) and 0.46 (II).

Fig. 6. Effect of pH on the adsorption of lysine by carboxyl cellulose.

The plot is for the average pH during the equilibration period and shows maximum adsorption at pH 7.0 with no binding when the carboxyl cellulose is not charged (pH 3.0) and no binding when the lysine is not charged (pH 10.0). Lysine was determined by the method of MOORE AND STEIN[11].

Peptides

An apparent difference in the ability of carboxyl cellulose and carboxymethyl cellulose to hold the tannin precipitable peptides responsible for formation of beer haze has been noted[12]. Carboxymethyl cellulose will adsorb all the peptides until it becomes overloaded, however, carboxyl cellulose allows 36 % of the tannin precipitables to come through. The phenomenon is not an effect of overloading and is thought to reveal a selectivity that reflects the difference in characteristics of the two materials as ion exchangers.

Other modifications

Periodate cleavage and chloride oxidation can be carried out on Whatman No. 1 filter paper to form ion exchange paper capable of binding cytochrome C and separation of its oxidized and reduced forms. A problem of keeping the paper intact and retaining dimensional stability is encountered due to the prolonged emersion of the paper in an aqueous medium. Placing the paper to be modified between two pieces of fiber glass screening gives sufficient support during the reaction and drying the paper on a photographic print dryer lends stability when the reaction is completed.

Sephadex, a high molecular weight dextran, may also be used as a base for

adding carboxyls. It does not react as readily under the conditions described as does cellulose and yields only 0.53 mequiv./g titrable carboxyl after 24 h exposure to periodate with subsequent chlorous acid oxidation. The resulting product is changed little in physical appearance by the reaction and it binds and permits chromatographic purification of cytochrome C by the system described for carboxyl cellulose.

Amino-cellulose

Attempts to place amino groups on the dialdehyde cellulose have been made with limited success. About 10 % of the substitution predicted on the basis of aldehyde content is achieved when dialdehyde cellulose, in the presence of Raney nickel, is reacted with liquid ammonia and catalytically hydrogenated for 4 h under 750 lb. pressure. Further substitution may be possible if a method allowing the mixing of the dry reactants under high pressure were used.

DISCUSSION

Three common methods of placing carboxyl groups on the cellulose polymer are: (1) substitution of a carboxyl-containing prosthetic group via an ether linkage to one of the free hydroxyl groups on the glucose sub-unit[1]; (2) conversion of cellulose sub-units from glucose to glucuronic acid by nitrogen tetroxide oxidation[13]; (3) periodate cleavage and subsequent chlorite oxidation of the resulting carbonyl groups on carbon atoms 2 and 3 of the glucose sub-units. The latter method produces a carboxyl ion exchange material with many of the properties of those presently in use, plus some properties which are unique. Unlike the first two methods, the synthesis is simple and requires a minimum of attention. The ease of handling the product and its intermediate allows synthesis of larger batches in the laboratory. The amount of substitution is low and can be controlled within fairly narrow limits permitting the production of batches with similar degrees of substitution. The one expensive reagent, potassium metaperiodate, can be economically regenerated[14] when larger amounts of carboxyl cellulose are required. Considering reagent cost and personnel time in synthesis, it is an inexpensive ion exchange material.

The difference in retention of tannin precipitable peptides by carboxyl cellulose and carboxymethyl cellulose suggests the existence of subtle differences in their adsorption properties. While carboxyl ion exchangers may behave in a similar manner with some proteins, the supporting structure and spacial placement of the carboxyl groups on ion exchange adsorbants appear to effect their ability to bind proteins and peptides. The effect of having adjacent carboxyl groups in some positions and carboxyl and carbonyl groups adjacent in other positions is unclear; however, the adjacent carboxyl groups have been useful in the chromatography of nickel and iron[15]. It is probably fair to say that the presence of the unique groupings function to limit the bonding potential of the carboxyl group making it more selective.

SUMMARY

A carboxyl cellulose ion exchange adsorbent can be synthesized by controlled periodate cleavage of SW-40-B Solkafloc followed by chlorous acid oxidation. The material is capable of separating the components of a cytochrome C preparation in

much the same way as IRC-50. The properties of carboxyl cellulose and the synthesis of carboxyl cellulose paper and carboxyl Sephadex are discussed.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART XII. AUTOMATIC ANALYSIS OF PHOSPHORUS-ANION MIXTURES BY ANION EXCHANGE CHROMATOGRAPHY

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Ion exchange chromatography can be made an automatic procedure by passing the effluent from the column through a system which continuously monitors the concentration of the solutes being separated. The detector for the monitoring system may be based on absorptiometric[1], conductivity[2], refractive index[3], flame photometric[4] or polarographic[5] methods and procedures. If radioactive tracers are used, continuous measurement of the effluent activity is made employing Geiger-Müller or scintillation counters[6] depending upon the counting efficiency required.

SPACKMAN, STEIN AND MOORE[1] have described a system which makes the analysis of amino-acids by ion exchange chromatography an automatic procedure. Essentially this involves mixing the column effluent with ninhydrin and continuously measuring the colour produced with a flowing colorimeter. Since the colour intensity for a given molar concentration depends on the amino-acid, this system has to be calibrated for each amino-acid. LUNDGREN AND LOEB[7] have described the automation of the anion exchange separation of condensed phosphates, originally described by GRANDE AND BEUKENKAMP[8], using the Technicon Autoanalyser. In this system, the column effluent containing condensed phosphates is pumped through a glass coil immersed in oil at 95° after being mixed with 6.66 *N* sulphuric acid, to hydrolyse the polyphosphates to orthophosphate. The liquid stream, now containing phosphorus only as orthophosphate, is passed through a dialyser and mixed with solutions of ammonium molybdate and hydrazine sulphate. The intensity of the molybdenum blue complex colour produced is measured continuously with a flowing colorimeter and since phosphorus is present only as orthophosphate the system does not have to be calibrated for each phosphorus anion.

We have modified the analytical system described by LUNDGREN AND LOEB[7] so that analysis of mixtures containing lower phosphorus anions, thiophosphates, amido- and imido-phosphates as well as polyphosphates is made a fully automatic procedure.

THE ANALYTICAL SYSTEM

In the analytical system, the phosphorus concentration in the sample stream is continuously monitored using a colorimetric procedure based on the molybdenum blue method for the determination of phosphorus. This method depends on the condensation of orthophosphoric and molybdic acids to give phosphomolybdic acids which, on

reduction, give an intensely blue coloured complex known as molybdenum blue, the intensity of the colour being proportional to the amount of phosphate ion incorporated in the complex. Since only orthophosphoric acid condenses with molybdic acid to form the complex acids, all the phosphorus present in the colorimetric system must be as orthophosphate. In the first part of the analytical system therefore, all the phosphorus must be quantitatively converted to orthophosphate and this may involve oxidation, hydrolysis, or both.

EXPERIMENTAL

The analytical system is based on the Technicon Autoanalyser and consists of a peristaltic proportionating pump to mix liquid streams in specified proportions, two double coil heating baths (with adjustable thermoregulators), a time delay coil, a flowing colorimeter fitted with silicon photocells and a potentiometric recorder (Elliott Dynamaster). The proportionating pump was fitted with 15 channel end blocks so that 15 liquid streams could be pumped simultaneously and by stretching tubes of various internal diameter between these end blocks, various liquid flow rates could be obtained.

The oxidation cycle

The effluent from the column is pumped at 0.42 ml/min and mixed with sodium hypochlorite solution (50 ml B.D.H. reagent grade sodium hypochlorite solution per litre) which is pumped at 1.2 ml/min (Fig. 1). Air is introduced at the point where the liquid streams meet so that the liquid stream is segmented to prevent diffusion. To en-

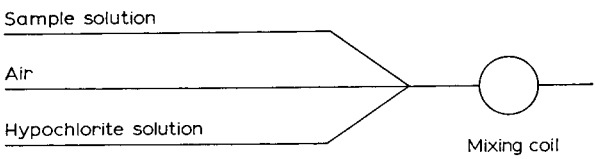

Fig. 1. The oxidation cycle.

sure that the liquid in each segment is homogeneous, the liquid stream is passed through a glass coil, positioned so that the axis of the coil is horizontal, where the pulsing motion of the segments ensures complete mixing of the liquid in each segment. The liquid stream then passes through a glass coil (40 ft. long), immersed in oil at 95°, and through a second glass coil (40 ft. long), at room temperature to effect the oxidation.

Owing to pressures which are built up inside these coils, especially in the one maintained at 95°, the flow rate of the liquid stream leaving the second coil is erratic and, if further reagents are introduced into this stream, the dilution will be erratic and valueless traces obtained. This difficulty can be overcome by passing only a part of the stream leaving the second coil through the pump at a constant rate so that, when it is mixed with further reagent streams, the dilution is uniform and reliable traces are obtained (Fig. 2).

Averaged over a fairly short interval of time, the sample stream recycled through the pump will be a constant fraction of that leaving the heating bath. Since the rate of change of phosphorus concentration in the column effluent is comparatively small, any errors introduced by the erratic flow rate of the sample stream leaving the heating

bath will be negligible. So that all the air will be lost before the sample stream is recycled through the pump, the pumping rate must be less than the lower limit of the

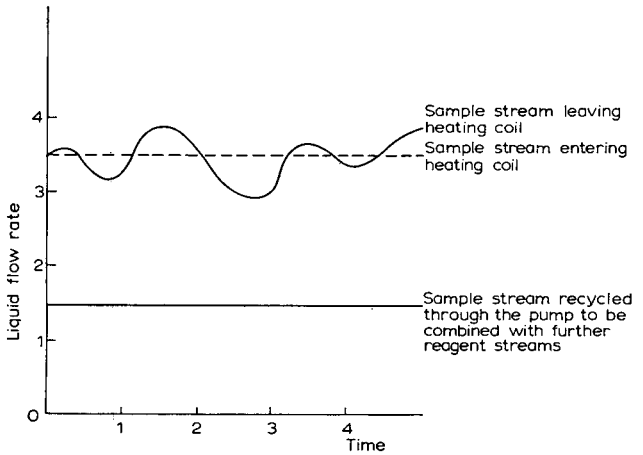

Fig. 2. Pumping rate traces.

flow rate of the stream leaving the heating bath. This is determined by trial and error. The average value of $(a + x)$ (see Fig. 3) will be equal to the rate at which liquid is being pumped into the heating bath.

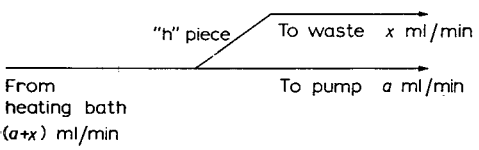

Fig. 3. Stream splitter for constant pumping speeds.

The hydrolysis cycle

The fraction of the sample stream retained in the analytical system is pumped at 0.32 ml/min and mixed with 10 *N* sulphuric acid containing 10 ml/l of saturated bromine water which is pumped at 1.2 ml/min. The liquid is resegmented with air and passed through a mixing coil before it enters a heating coil (2 glass coils, each 40 ft. long) maintained at 95°. After leaving the heating coil the liquid stream is cooled in a water-jacketed coil.

The colorimetric cycle

Once again in order to counteract surging in the sample stream, only a part (0.32 ml/min) is retained in the analytical system. This is mixed with a 1.25 % ammonium molybdate solution in 1.00 *N* sulphuric acid (0.80 ml/min) and segmented with air. After passing through a mixing coil, 0.05 % hydrazine sulphate solution is injected at 1.6 ml/min into the sample stream through an 'h' piece. This stream is pumped through a mixing coil and into a heating coil (40 ft. long) maintained at 95° to effect the development of the phosphomolybdenum blue complex.

The sample stream next passes through a cooling coil and then through the flowing colorimeter (silicon photocells, 801 mμ filters and a 10 mm flow cuvette). The percentage transmittance of the liquid stream flowing through the cell is read from a recorder chart (see Fig. 4). The flow time of the complete system is approximately one hour.

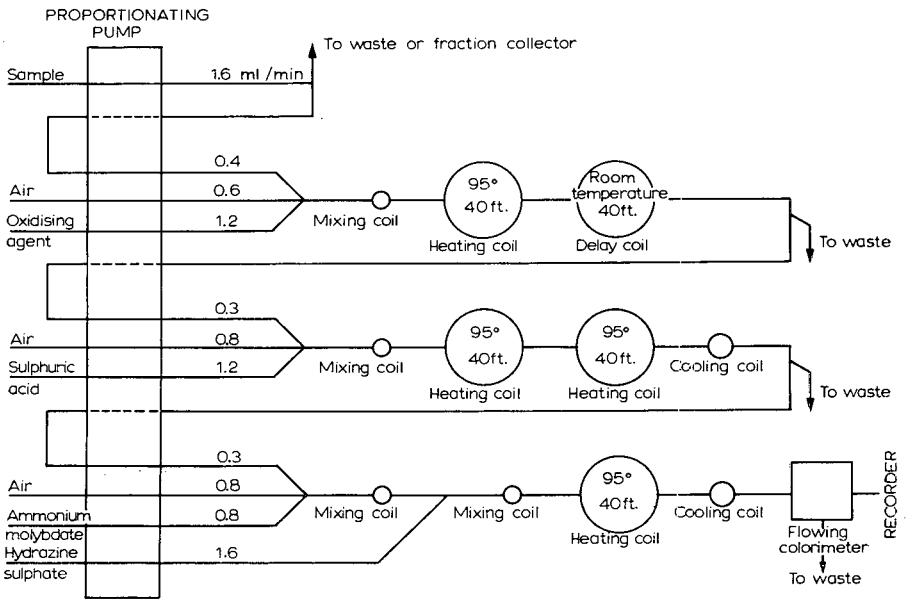

Fig. 4. Technicon Autoanalyser Module for phosphate anions.

Reagents

(*a*) *For the quantitative conversion of all phosphorus anions to orthophosphate.* Polyphosphates are readily hydrolysed to orthophosphate by strong acids and so 10 *N* sulphuric acid was chosen as the reagent for the hydrolysis cycle.

The choice of reagent in the oxidation cycle is more difficult because some lower oxyanions of phosphorus are only oxidised in neutral solution and others only in acid solution. In order to oxidise any anions which may only be oxidised in strong acid solution, saturated bromine water (10 ml/l) was added to the sulphuric acid used as reagent in the hydrolysis cycle. Several oxidising agents were used as reagents in the oxidation cycle, but only a solution of sodium hypochlorite (50 ml B.D.H. reagent grade sodium hypochlorite solution per litre) was found to quantitatively convert anions containing P–P bonds to orthophosphate.

To show that this reagent system quantitatively converts phosphorus anions to orthophosphate, samples of each anion to be studied were pumped through the analytical system and the optical density of the final sample stream was compared with that of an equivalent sample which had been converted to orthophosphate externally. External conversion to orthophosphate was effected by boiling with bromine water (10 ml of a saturated solution) in neutral solution for 1 hour, and then with bromine water (10 ml saturated solution) and 10 *N* sulphuric acid (10 ml) for a further hour.

The sample solutions were made 0.1 *M* with respect to potassium chloride and buffered to (1) pH 6.8, and (2) pH 11.4, in order to simulate anions in the column effluent.

The agreement between the optical density of the sample internally converted to orthophosphate, and that of the external standard (Table I) demonstrates that the

TABLE I

DIFFERENCE BETWEEN THE OPTICAL DENSITY OF THE EXTERNAL STANDARD AND THAT OF THE INTERNALLY CONVERTED SAMPLE

Anion	*Difference at*	
	pH 6.8	*pH 11.4*
H_2PO_2	+ 0.5 %	+ 0.5 %
HPO^2_3	− 1.5 %	+ 0.9 %
PO_3S^{3-}	0.0 %	− 0.5 %
$PO_2S_2^{3-}$	+ 1.0 %	0.0 %
Pyrophosphate	− 0.5 %	0.0 %
Hypophosphate	0.0 %	—
Diphosphite	− 0.5 %	—
P^4–P^3–P^4	− 1.5 %	—
Monoamidophosphate	0.0 %	0.0 %
Trimetaphosphate	− 0.5 %	− 1.0 %

reagent system described is satisfactory for the quantitative conversion of these inorganic phosphates and lower phosphorus anions to orthophosphate, and we see no reason why it should not be satisfactory for other more complicated inorganic phosphorus anions.

(*b*) *For the colorimetric determination of phosphorus by the molybdenum blue method.* For the estimation of orthophosphate in the colorimetric system, the modification of the molybdenum blue method described by LUNDGREN[9] was used. In this system, the phosphomolybdic acid (formed by condensation of phosphoric acid and molybdic acid) is reduced by hydrazine sulphate in acid solution at 95°, to the phosphomolybdenum-blue complex.

(i) Ammonium molybdate solution: 1.25 % w/v ammonium molybdate in 1.0 *N* sulphuric acid.

(ii) Hydrazine sulphate solution: 0.05 % w/v aqueous hydrazine sulphate solution.

Calibration of the module

The module was calibrated by sampling solutions of sodium dihydrogen phosphate (Analar R) varying in concentration from 1 to 50 p.p.m. of phosphorus through the module.

A plot of absorbance of the final solution against concentration of phosphorus (Fig. 5) shows that Beer's law holds up to a concentration of 25 p.p.m. phosphorus in the sample solution (the transmittance corresponding to the limit is 75 %). Owing to the design of the module, only a fraction of the phosphorus entering the module passes through the colorimetric cycle, the remainder going to waste. In fact, therefore, Beer's law only holds within a much narrower range of concentration of phosphorus.

If absolute figures for the amount of phosphorus inany sample are required, it is necessary to run a standard with each batch of samples, because dilution factors vary

from day to day (owing to stretching of the manifold tubes). In the work described relative rather than absolute figures for phosphorus concentration were required, and so day to day calibration of the module was not necessary.

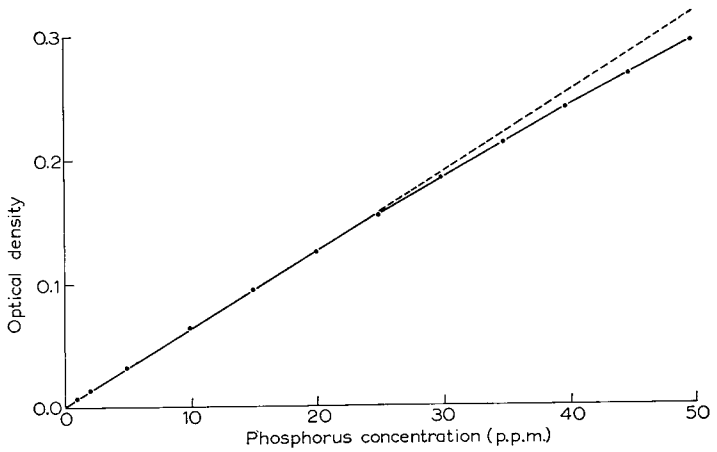

Fig. 5. Calibration graph for phosphorus as Molybdenum Blue.

The absorption spectrum of the final solution

The absorption spectrum of the molybdenum-blue complex obtained when a solution containing 100 p.p.m. phosphorus is sampled through the module was measured using a Unicam SP. 500 spectrophotometer. The spectrum indicates that the maximum absorption is at 817 mμ and hence 801 mμ filters (supplied by Technicon Instruments Ltd.), and silicon photocells were used in the colorimeter.

Anion-exchange chromatography

The ion-exchange columns were prepared in an identical manner to that described in previous publications[10,11], while the eluant gradients were obtained by the method described previously[7,8]. Without altering the analytical system, the column flow rate can be varied above a minimum of 0.42 ml/min, by pumping the column effluent at the required rate and rejecting all but that amount at a glass T-piece inserted in the liquid stream (see Fig. 6). The maximum permissible flow rate is governed by the porosity of the column, since pumping at too great a speed causes cracks and air-bubbles in the column.

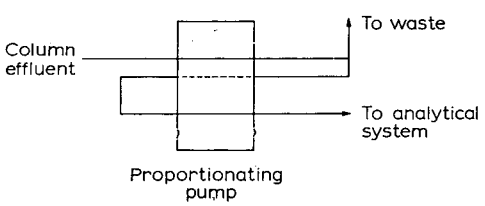

Fig. 6. Sample stream splitter.

The amount of phosphorus which can be applied to the column is limited by:

(i) the maximum load for which efficient chromatography can take place;

(ii) the fact that the minimum transmittance of the effluent must not be less than 75 %, otherwise Beer's law is not obeyed, and the calculated value of the phosphorus concentration would be subject to a systematic error.

Normally, the second factor is the limiting one, and thus the maximum load for any phosphorus anion depends on the time over which it is eluted from the column. (This will quite naturally depend on the dimensions of the column, the eluting gradient, and the affinity of the resin for the anion.)

Chromatographic separations

The following separations described in Table II and shown in Figs. 7 and 8 are typical of the results obtained.

TABLE II

SEPARATION DETAILS OF VARIOUS PHOSPHATE ANIONS

Figure No.	*Column dimensions*		*Resin Dowex-1*	*Temp.* (°C)	*pH*	*Flowrate* (*ml/min*)
	Length (*cm*)	*Diameter* (*cm*)				
7	50.0	1.5	× 8% D.V.B.	18	6.8	1.6
8	14.0	1.0	× 10% D.V.B.	~2*	11.4	1.6

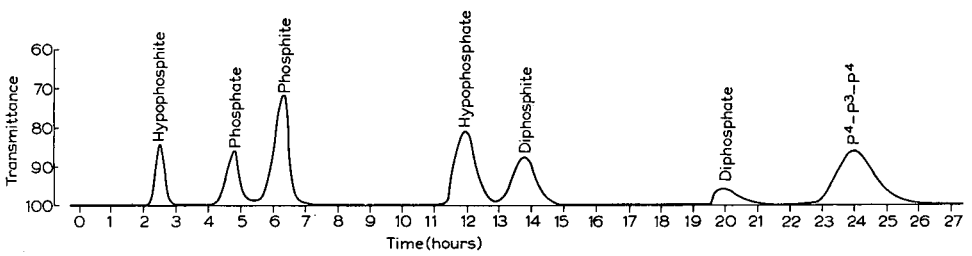

Fig. 7. Separation of lower phosphorus-containing anions.

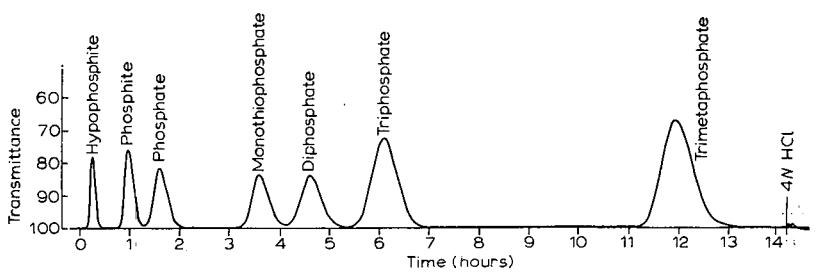

Fig. 8. Separation of simple and complex phosphorus-containing anions.

Quantitative evaluation of elution curves

The recorder pen of the Autoanalyser traces the variation with time of the transmittance rather than the absorbance of the solution in the colorimetric system, which means that elution curves cannot be quantitatively interpreted, simply by measuring peak areas with a planimeter. There are, however, several good approximation methods by which the peak area on an absorbance scale can be evaluated from the Autoanalyser trace:

(i) Assuming that the variation of phosphorus concentration in the column effluent is a Gaussian function of time.

(ii) Division of each of the peaks into narrow strips of equal width followed by calculation and summation of strip absorbances.

Since the phosphorus anions are monitored as orthophosphate in the colorimetric cycle, the distribution of phosphorus amongst the species represented by the peaks in the elution trace is simply given by the distribution of peak areas on an absorbance scale, provided that the absorbance of the molybdenum blue complex is within the limit of linearity with concentration of phosphorus.

According to Beer's law:

$\log_{10} T_0/T = \text{Absorbance} = k.c$

where:

T_0 is the transmittance of the reagent blank;

T is the transmittance of the solution in the colorimetric system;

c is the concentration, in this case of the molybdenum-blue complex;

k is a constant depending on the extinction coefficient of the absorbing species and the length of the absorbance cell.

It should be noted that T is the true percentage transmittance of the solution. (When a range expansion factor other than 1 is used, the value for transmittance obtained from the recorder chart scale, T^c, must be corrected to the equivalent value for an expansion factor of unity.) Let the expansion factor be x (for the Range Expander unit supplied x may be 1, 2, 4 or 10).

The corrected transmittance is given by

$$T_b{}^c \left(1 - \frac{1}{x}\right) + \frac{T^c}{x}$$

where T^c is the value of the transmittance on the chart scale, and $T_b{}^c$ is the value of the transmittance on the chart scale for which balance was obtained.

(*i*) *Calculation of absorbance peak areas assuming the elution peaks to be Gaussian.* The phosphorus load (L) corresponding to an elution peak is given by:

$$L = \int_{-\infty}^{+\infty} c.\, \mathrm{d}t$$

where c = concentration.

Since flowrates in this system must be assumed to be constant, the variable volume may be replaced by time. When the elution peak is assumed Gaussian, the variation of concentration with respect to time is given by the formula

$$c = A \cdot \mathrm{e}^{-h^2 (t-m)^2}$$

where:

A is the maximum value of c for the peak,

m is the value of t corresponding to the peak maximum (retention time),

t is time,

h is a measure of the time interval during which the concentration is greater than $A/2$.

If such a time interval is defined by b, then,

$$h = \frac{2}{b}\sqrt{\log 2}$$

For a given phosphorus load, the values of A, m, and h will depend on the column parameters, the eluant gradient, and the ionic species being eluted.

$$\therefore L = \int_{-\infty}^{+\infty} e^{-h^2(t-m)^2}\, c.dt$$

$$= \frac{A\sqrt{\pi}}{h}$$

$$= Ab\sqrt{\frac{\pi}{4\log 2}} = \text{const. } A.b$$

Since the column load can be adjusted so that the phosphorus concentration in the column effluent is such that the concentration of the molybdenum blue complex remains within the limit of linearity with absorbance, c may be redefined as the absorbance. Thus, the phosphorus load corresponding to an elution peak will be proportional to the product of the peak absorbance, and the time interval during which the absorbance is greater than one half the maximum absorbance of the peak. These two parameters can readily be obtained from the Autoanalyser trace by using the general conversion formula:

$$\text{Absorbance} = T_b^c\left(1 - \frac{1}{x}\right) + \frac{T^c}{x}$$

To use this method for the calculation of the phosphorus load, L:

(a) the transmittance of the base-line T_b^c and peak maximum T_p^c are measured and corrected to their equivalents for an expansion factor of unity, say T_0 and T_p respectively. The value $\log_{10} T_0/T_p$ is the absorbance value of the peak maximum A;

(b) the transmittance (when $x = 1$) corresponding to an absorbance half that of the peak maximum is determined using the identity

$$\log_{10} T_p/2 = \log_{10} T_0 - \tfrac{1}{2}\log_{10} T_0/T_p$$

The width of the peak on the recorder trace when the transmittance ($x = 1$) is equal to $T_p/2$ is measured. This is b. The distribution of phosphorus among the peaks of an elution trace is then simply obtained by measuring the distribution of the product, $A.b$, for the peaks.

(*ii*) *Division of the peaks into narrow strips of equal width, followed by calculation and summation of absorbances.* Using the same nomenclature as under (*i*), an approximation to the absorbance peak area is obtained by dividing the peak obtained or the recorder chart into narrow strips, each of equal width, a, and measuring the transmittance, T, at the sides of each strip.

By adding the values of $\log_{10}(T_0)-\log_{10}(T)$ for all the strips comprising the peak, a value is obtained which, to a first approximation, is proportional to the corresponding load of phosphorus; the constant of proportionality being the same for all peaks in the elution trace, the degree of approximation being determined by the number of strips into which the peak is divided.

$$L = K \sum_{t=t_1}^{t=t_2} \log T_0/T$$

where t_2 and t_1 are times for the end and beginning of the peak respectively.

T is the transmittance at time t, and K includes the factor a.

For an expansion factor, x,

$$L = K \sum_{t=t_1}^{t=t_2} \log_{10} [T_b{}^c (x - 1) + T_0{}^c]/[T_b{}^c (x - 1) + T^c]$$

(When the base-line transmittance $T_0{}^c$ is not the same on either side of the peak, the average of $T_0{}^c$ at t_1 and $T_0{}^c$ at t_2 is substituted for $T_0{}^c$.)

This method of calculating the absorbance peak area is obviously very tedious, but it can be made a useful method by using a computer to evaluate the general expression for the phosphorus load given the values of $T_0{}^c$ and T^c for each peak. For the results which follow, an I.B.M. 1620 machine was programmed to process these data; the programme details together with more observations concerning this method will be published in a subsequent paper. It was considered necessary in this paper to outline the type of results which can be obtained comparing methods (i) and (ii).

RESULTS

To test these methods, a solution containing sodium orthophosphate and orthophosphite was prepared by weighing, and several aliquots of this solution subjected to separation on an ion-exchange column, under the conditions given in Table II and Fig. 7. Each run was analysed, and the results are given in Tables III and IV, which are a

TABLE III

CALCULATION OF THE PHOSPHATE TO PHOSPHITE RATIO ASSUMING THAT THE ELUTION PEAKS ARE GAUSSIAN*

Run No.	*Peak absorbances*		*Peak widths at half height*		*Ratio* P^5/P^3
	Phosphite	*Phosphate*	*Phosphite*	*Phosphate*	
1	0.0800	0.0669	19.5	47.1	2.02
2	0.0705	0.0596	20.9	51.0	2.06
3	0.0713	0.0602	19.8	50.2	2.14
4	0.0738	0.0617	20.0	50.8	2.12
5	0.0770	0.0656	20.3	50.0	2.10
6	0.0763	0.0607	20.9	52.3	1.99
7	0.0770	0.0632	19.9	51.0	2.10
8	0.0753	0.0613	20.1	51.2	2.07
9	0.0731	0.0605	21.0	52.3	2.06
			20.3 ± 0.5	50.7 ± 1.5	2.07 ± 0.04

* These results were obtained with K. W. C. BURTON, whose help is gratefully acknowledged.

TABLE IV

"STRIP" SUMMATION METHOD USING AN I.B.M. 1620

Run No.	*Absorbance phosphite*	*Absorbance phosphate*	*Ratio P^5/P^3*
1	1.4935	3.1092	2.082
2	1.4359	2.9619	2.063
3	1.3885	2.9622	2.133
4	1.4450	3.3039	2.096
5	1.5433	3.2115	2.081
6	1.5400	3.1100	2.019
7	1.4890	3.1183	2.094
8	1.4981	3.0649	2.046
9	1.5184	3.1271	2.059
			2.07 ± 0.03

direct comparison of methods (i) and (ii) applied to each separation. The weighed ratio of phosphate to phosphite was 2.07:1, and the results in the final column of each table compare very favourably with this value. These results, together with the programme, will be discussed at greater length, with many other observations on the system in a subsequent paper.

ACKNOWLEDGEMENTS

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SUMMARY

The development of a system, based on the Technicon Autoanalyser, is described for the quantitative determination of the phosphorus concentration in the column effluent from ion-exchange column separation of phosphorus anions. Quantitative interpretation of the elution peaks by the Gaussian approximation and by integration of peak areas are compared.

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SUR LES COMPLEXES DE L'IRIDIUM TÉTRAVALENT ET DE L'IRIDIUM TRIVALENT AVEC LE BROMURE D'ÉTAIN (II)

ÉTUDE CHROMATOGRAPHIQUE

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(Reçu le 9 avril 1964)

Les complexes colorés obtenus en faisant réagir le bromure ou le chlorure d'étain (II) avec Pt, Pd, Rh, Os, Ru et Ir ont fait l'objet de plusieurs études et récemment on a proposé une configuration de ces complexes où l'étain était directement lié au métal central[1].

Utilisant la technique de la chromatographie sur papier LEDERER ET SHUKLA[2] ont montré que la plupart de ces éléments, dans une solution de HBr, réagissaient avec l'étain bivalent en formant un complexe unique; l'iridium faisait exception donnant, dans les mêmes conditions, un ensemble de complexes séparables chromatographiquement.

Cette observation est en contradiction avec les conclusions des travaux de BERMAN ET MCBRYDE[3] et de PANTANI ET PICCARDI[4] qui ont eu pour objet la mise au point d'une méthode spectrophotométrique de détermination quantitative de l'iridium.

De là, l'intérêt que peut représenter une étude un plus peu détaillée de la réaction qui a lieu dans une solution de HBr entre l'iridium et le bromure d'étain (II). C'est dans ce but que nous avons entrepris ce travail. Nous avons utilisé dans notre recherche la chromatographie par adsorption sur papier, la spectrophotométrie et l'électrochromatographie. Des essais chromatographiques préliminaires confirment les résultats de LEDERER ET SHUKLA[2], et montrent que le comportement de l'iridium-(IV) est indépendant des produits commerciaux utilisés, tels que H_2IrCl_6, $(NH_4)_2$-$IrCl_6$ et Na_2IrCl_6; l'Ir (III), produit commercial K_3IrCl_6, dans les mêmes conditions, diffère de l'Ir (IV) uniquement par l'absence d'une tâche lente rosâtre. Cependant, les spectres d'absorption de solutions fait avec de l'Ir (III) et de l'Ir (IV), réalisés avec des solutions très diluées, présentent la même forme avec des pics aux mêmes longueurs d'onde. De plus, les produits de la réaction sont considérablement influencés par le rapport Ir/Sn, par la durée du chauffage a 100° ainsi que par le vieillissement à température ambiante.

ÉTUDE CHROMATOGRAPHIQUE

(1) *Partie expérimentale*

Nous avons utilisé le papier Whatman No. 3 MM et le développement à été fait dans des récipients en verre (25 cm × 14 cm de diamètre) soigneusement fermés; nous avons employé la technique de la chromatographie ascendante utilisant comme

éluant une solution de $SnBr_2$ en HBr préparée en dissolvant 50 g de Sn dans 500 ml de HBr conc. (48 %, d = 1.49) et en diluant le tout à 1 l. Nous avons ainsi obtenu une solution 0.42 *M* en Sn(II) et 4.47 *M* en Br^-.

(2) *Résultats*

En mélangeant l'Ir avec un excès de $SnBr_2$ en HBr et en chauffant au bain-marie durant quelques minutes, on obtient par chromatographie une tâche rosâtre (R_F = 0.16) spécifique à l'Ir(IV), se formant également à froid, et trois autres tâches, communes à l'Ir(III) et (IV): une tâche jaune citron ($R_F \simeq 0.87$), et deux tâches d'un jaune canari foncé ayant respectivement des R_F d'environ 0.57 et 0.23 (Fig. 1).

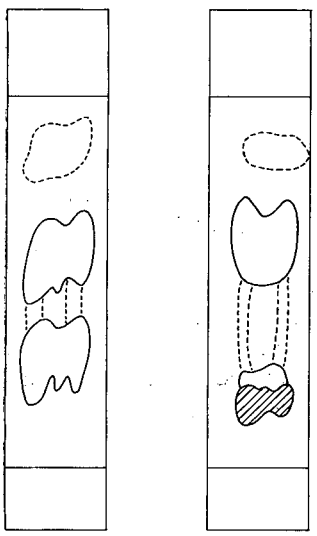

Fig. 1. Chromatogrammes de solutions contenant de l'Ir avec un excès de Sn(II,) chauffées durant 5 min au bain-marie bouillant (essais préliminaires). (a) solution contenant de l'Ir(III); (b) solution contenant de l'Ir(IV).

Cependant ces tâches ont une intensité variable et peuvent même disparaître selon les conditions d'expérience. Nous avons étudié trois facteurs qui ont une influence sensible sur cet équilibre: le rapport Ir/Sn, la durée du chauffage et le vieillissement à température ambiante.

Effet du rapport Ir/Sn et de la durée du chauffage. Nous avons préparé des solutions bromhydriques d'Ir(III) et d'Ir(IV) contenant le Sn(II) suivant des rapports molaires croissants allant de 1:1 jusqu'à 1:84. Nous avons chromatographié ces solutions après 2, 5, 30 et 90 min de chauffage au bain-marie bouillant. Les chromatogrammes ont montré qu'en prolongeant la durée du chauffage et en augmentant la quantité relative de l'étain, la formation de la tâche lente jaune canari était favorisée et celle-ci devenait prépondérante après 90 min de chauffage. La tâche rapide jaune citron, quels que fussent les rapports Ir/Sn disparaissait toujours après 90 min. Cependant quand le rapport Ir/Sn était supérieur a 1:6, elle disparaissait même après 30 min de chauffage. La tâche rapide jaune canari et la tâche

lente de teinte rosâtre, indépendemment du rapport Ir/Sn, diminuaient d'intensité en prolongeant le chauffage mais persistaient après 90 min (Figs. 2 et 3).

Effet du vieillissement à température ambiante. L'étude de ce facteur a été fait dans un cas, avec des solutions chauffées après avoir été laissées pendant 2 jours à température ambiante, et dans un autre cas, avec des solutions qui avaient été chauffées durant 90 min immédiatement après leur préparation.

Dans le 1er cas, les chromatogrammes ont montré la persistance de la tâche jaune citron après 90 min de chauffage, indépendemment du rapport Ir/Sn, et l'absence totale de la tâche lente jaune canari (Figs. 4 et 5).

Dans le 2ème cas nous avons vu qu'en général la tâche lente jaune canari perdait d'intensité au profit de la tâche rapide de même couleur; ces deux tâches apparaissaient très souvent reliées par des comètes et parfois elles se confondaient en une seule grande tâche. Au bout d'un certain temps, il ne restait que 2 tâches, l'une rapide jaune canari, et l'autre lente de teinte rosâtre. Cependant le temps durant lequel s'accomplit ce processus varie considérablement. En effet les solutions employées au cours de nos essais préliminaires ont révélé la persistance de la tâche lente jaune canari même après 72 jours, sa disparition n'advenant qu'au bout de 76 jours; tandis que d'autres solutions, contenant à peu près le même rapport Ir/Sn, ont montré la disparition de la tâche jaune déjà au bout de 10 jours (Figs. 6 et 7).

Enfin en développant avec un éluant vieilli, nous avons remarqué l'apparition d'une nouvelle tâche marron brunâtre (R_F 0.76) qui est probablement un produit d'oxydation, et, pour la première fois, l'absence de la tâche rosâtre (Fig. 8).

Les résultats de cette étude chromatographique ne donnent qu'une idée approximative du mécanisme de la réaction; cependant il est clair qu'il se forme une série de complexes instables, en équilibre entre eux. Cette instabilité empêche leur isolement par élution des chromatogrammes.

ÉTUDE SPECTROPHOTOMÉTRIQUE

(1) *Partie expérimentale*

Nous avons utilisé un spectrophotomètre enregistreur DK_2, deux cellules de quartz de 1 cm de largeur, préalablement étalonnées, et nous avons préparé une solution 0.1 *M* en Sn(II) et 4.30 *M* en HBr contenant 6 μg d'Ir/ml, semblable à celle employée par PANTANI ET PICCARDI[4].

(2) *Résultats*

Les solutions d'Ir(III) et d'Ir(IV) chauffées durant 2 min ont présenté un maximum d'absorption à 400 mμ, mais après 90 min de chauffage ces spectres ont changé de forme et le pic s'est déplacé à 370 mμ. Des solutions préalablement chauffées durant 90 min et laissées vieillir, ont donné après deux jours, des spectres modifiés avec un pic à 380 mμ (Fig. 9).

En confrontant les résultats obtenus chromatographiquement avec ceux obtenus spectrophotométriquement, il semble probable que le spectre fait après 2 min de chauffage, identique à celui obtenu par PANTANI ET PICCARDI[4], corresponde à la tâche rapide jaune citron. De même celui ayant un seul pic à 370 mμ correspondrait à la tâche lente jaune canari et celui indiquant un maximum d'adsorption à 380 mμ, à la tâche rapide jaune canari. Pour la tâche rose, qui ne trouve pas son correspondant

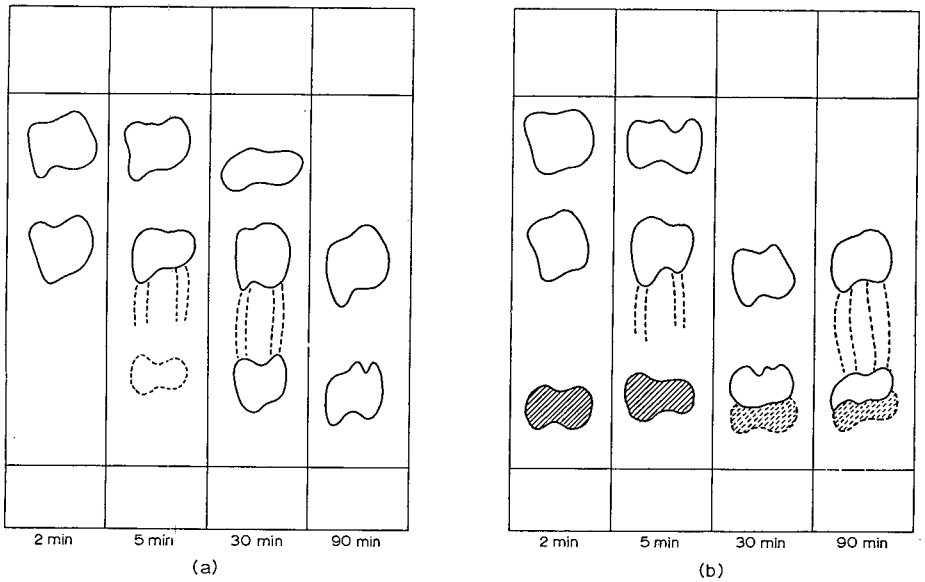

Fig. 2. Chromatogrammes de solutions chauffées au bain-marie bouillant durant 2, 5, 30 et 90 min. (a) Ir(III)/Sn(II) = 1:5 (rapport molaire); (b) Ir(IV)/Sn(II) = 1:5 (rapport molaire).

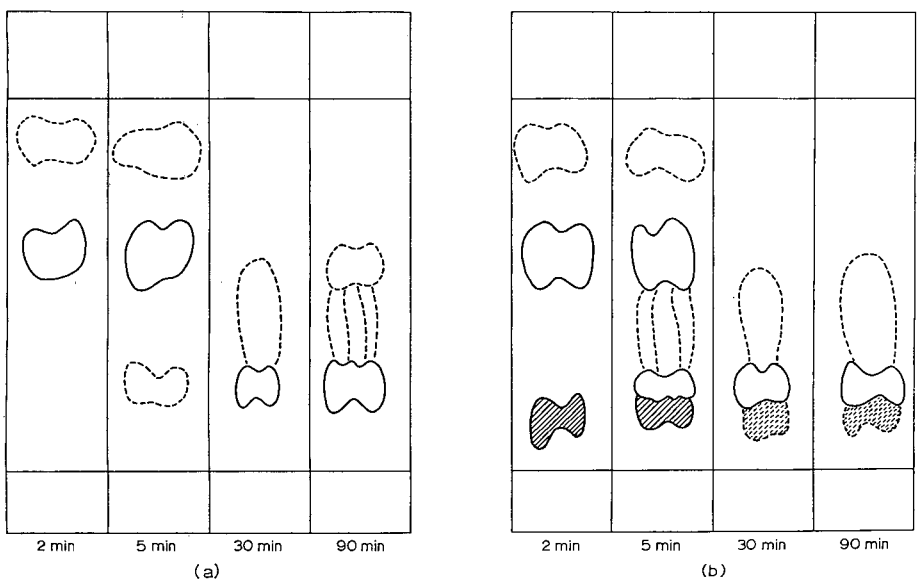

Fig. 3. Chromatogrammes de solutions chauffées au bain-marie bouillant durant 2, 5, 30 et 90 min. (a) Ir(III)/Sn(II) = 1:30 (rapport molaire); (b) Ir(IV)/Sn(II) = 1:30 (rapport molaire).

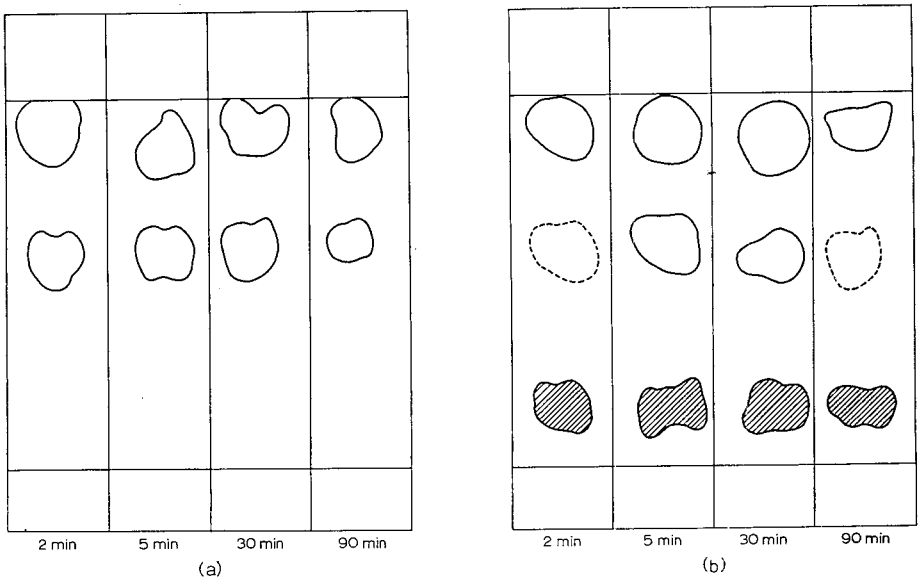

Fig. 4. Chromatogrammes de solutions laissées à température ambiante pendant 2 jours et chauffées ensuite durant 2, 5, 30 et 90 min. (a) Ir(III)/Sn(II) = 1:5 (rapport molaire); (b) Ir(IV)/Sn(II) = 1:5 (rapport molaire).

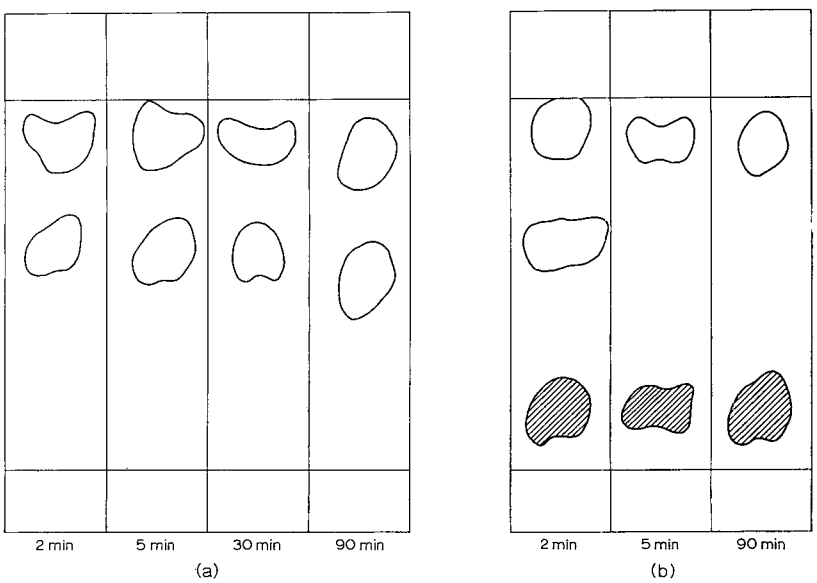

Fig. 5. Chromatogrammes de solutions laissées à température ambiante pendant 2 jours et ensuite chauffées. (a) Ir(III)/Sn(II) = 1:17, chauffage effectué durant 2, 5, 30 et 90 min; (b) Ir(IV)/Sn(II) = 1:17, chauffage effectué durant 2, 5 et 90 min.

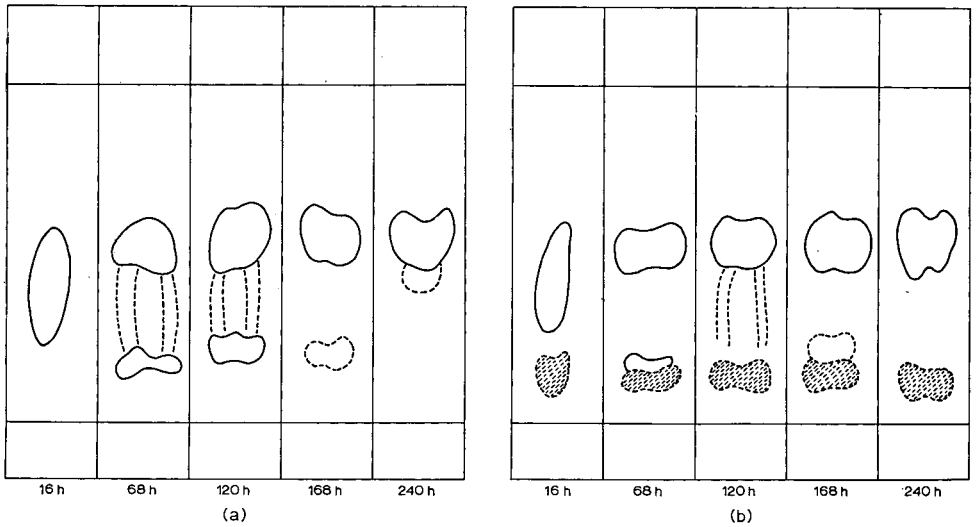

Fig. 6. Solutions chauffées durant 90 min immédiatement après leur préparation; chromatogrammes effectués respectivement après 16 h, 68 h, 120 h, 168 h et 240 h de vieillissement à température ambiante. (a) Ir(III)/Sn(II) = 1:30 (rapport molaire); (b) Ir(IV)/Sn(II) = 1:30 (rapport molaire).

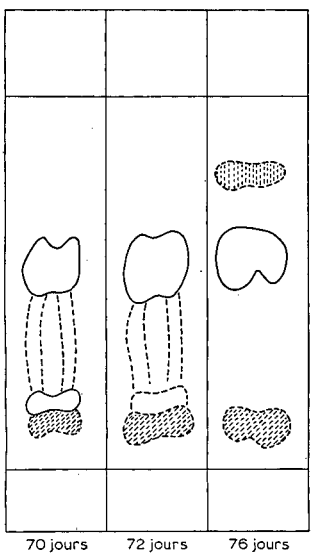

Fig. 7. Une solution contenant de l'Ir(IV) avec un excès de Sn(II); chromatogrammes effectués respectivement après 70 jours, 72 jours et 76 jours de vieillissement à température ambiante.

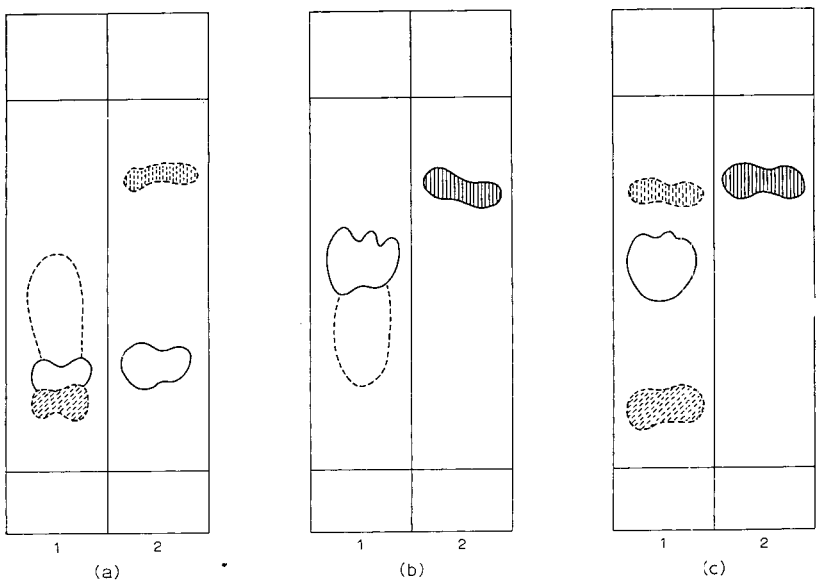

Fig. 8. Conséquences de l'emploi d'un éluant vieilli sur le développement des chromatogrammes de trois solutions différentes. (a) Solution contenant de l'Ir(IV) avec un excès de Sn(II); (1) chromatogramme effectué après 30 min de chauffage en développant avec un éluant frais; (2) chromatogramme obtenu en développant avec un éluant vieilli. (b) Solution contenant l'Ir(III) et le Sn(II- dans un rapport molaire de 1:30, chauffage pendant 90 min et vieillissement de 264 h à température ambiante; (1) chromatogramme effectué en utilisant un éluant frais; (2) chromatogramme obe tenu avec un éluant vieilli. (c) Solution contenant l'Ir(IV) et le Sn(II) dans un rapport molaire de 1:30; conditions d'expériences et exposition des chromatogrammes obtenus identiques au cas (b).

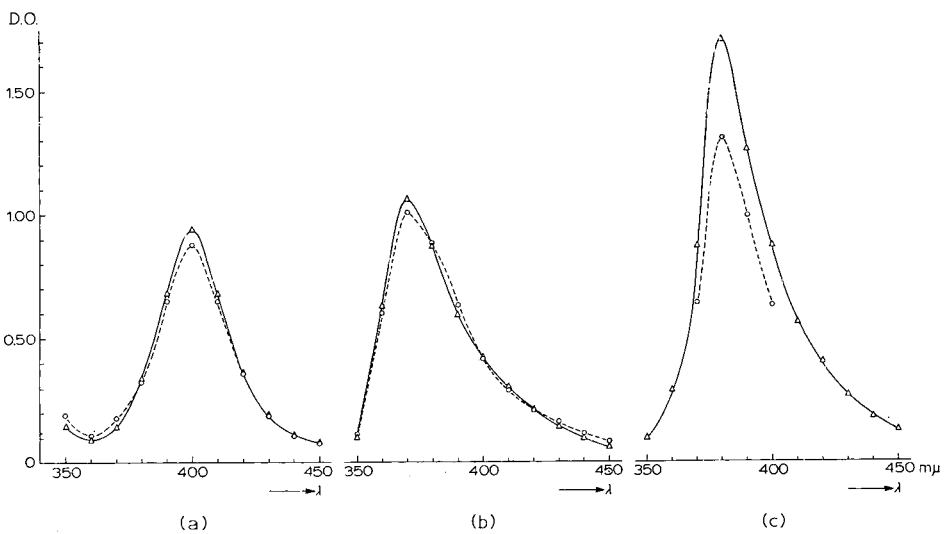

Fig. 9. Spectres des solutions contenant de l'Ir(III) et de l'Ir(IV). (a) Solutions chauffées durant 2 min immédiatement après avoir été préparées. (b) Solutions chauffées durant 90 min immédiatement après avoir été préparées. (c) Les mêmes solutions utilisées pour obtenir la Fig. 9b ont été laissées vieillir pendant deux jours.

en spectrophotométrie, il faut remarquer que les conditions d'expérience dans l'une et l'autre technique n'étaient pas rigoureusement identiques étant donné que les solutions étudiées spectrophotométriquement étaient nécessairement très peu concentrées en Ir.

ÉTUDE ÉLECTROCHROMATOGRAPHIQUE

(1) *Partie expérimentale*

Nous avons utilisé des bandes de papier Whatman No. 1 qui, tout en ayant les mêmes caractéristiques chromatographiques du papier Whatman No. 3 MM présentent l'avantage, pour une tension éléctrique donnée, de pouvoir opérer avec une intensité de courant plus basse. D'autre part, les produits de la réaction étant influencés par la température, nous avons placé la bande de papier sur une plaque creuse en cuivre parcourue intérieurement par un courant d'eau. Nous avons utilisé des électrodes de platine et un électrolyte 0.105 *M* en Sn(II) et 1.1 *M* en HBr. Le courant a été fait passer pendant 1.30 h sous une tension électrique de 100 V.

(2) *Résultats*

L'étude électrochromatographique a reproduit exactement la séquence des chromatogrammes. Tous les produits de la réaction ont migré vers l'anode. Il s'agit donc de substances anioniques, retardées par adsorption, à mobilités similaires.

CONCLUSIONS

Il semble donc que le composé étudié par BERMAN ET MCBRYDE[3] soit, dans dee solutions à concentration raisonnable en iridium, un produit intermédiaire; anioniqus en équilibre instable avec d'autres complexes, eux aussi, anioniques.

L'étain réagissant avec l'Ir peut aussi bien le réduire que le complexer; nous ne pouvons pas, pour le moment, établir lequel des deux phénomènes prévaut et dans quelles conditions; d'autre part, en assumant pour les complexes Ir–Sn une configuration avec un atome d'Ir central entouré d'un ou de plusieurs (jusqu'à six) groupes $SnCl_3^-$, on ne voit pas comment, le vieillissement à température ambiante, le rapport Ir/Sn, et le prolongement du chauffage, peuvent déterminer l'ordre dans lequel s'opère les substitutions du Cl avec les groupes $SnCl^-_3$ dans les radicaux $IrCl_6^{2-}$ et $IrCl_6^{3-}$. En d'autres termes il serait intéressant pouvoir isoler ces complexes et en étudier la structure. Des éclaircissements à ce sujet pourront être probablement fournis au terme d'un travail que nous sommes en train d'entreprendre.

REMERCIEMENTS

Nous tenons à remercier très vivement Mr. M. LEDERER qui nous a proposé ce travail et nous a constamment suivi et éclairé; nous remercions aussi Mme L. OSSICINI pour ses précieux conseils concernant la présentation du travail.

RÉSUMÉ

On a étudié la réaction entre l'Ir et le $SnBr_2$ dans une solution bromhydrique au moyen de la chromatographie par adsorption sur papier, la spectrophotométrie et l'électrochromatographie.

SUMMARY

A study was made of the reaction between Ir and $SnBr_2$ in a hydrobromic acid solution, by means of adsorption chromatography on paper, spectrophotometry and paper electrophoresis.

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Notes

A trap for the collection of a series of fractions in the preparative scale gas chromatography of high molecular weight compounds*

We have been experimenting at this laboratory with the method of preparative scale gas–liquid chromatography for the separation and isolation of pure samples of methyl esters of the polyenoic acids of marine oils. Runs have been carried out on large polyester columns with injected samples of up to 3 g of mixed esters at temperatures around 200° and with nitrogen carrier gas flow rates of 3 l/min. It was found in our earliest experiments that under these conditions ester was eluted from the column as an aerosol which defied quantitative condensation in liquid air traps. A study of the literature indicated that the problem of aerosol formation in the use of this technique was well known and its cause recognised[1]. A number of devices for overcoming it have been reported, the best known of these probably being the VOLMAN collector[2], in which the formation of a persistent aerosol is prevented by passing the effluent through a large temperature gradient. A variation on the VOLMAN trap has also been reported[3]. Other methods based on electrostatic precipitation[4] and re-vaporizing the aerosol in a single hot zone followed by secondary condensation in a falling temperature gradient[5] have also been published. A method recently described is based on passage of the column effluent through a number of consecutive hot and cold zones, the trap being constructed by simple modification of a reflux coil condenser[6].

Not one of these devices entirely fulfilled our immediate requirements, which included simplicity of construction, ready availability of parts and, probably most important, the suitability for collection of a series of fractions at timed intervals with rapid removal of the fraction from the trap, leaving it uncontaminated at the end of each period. This last requirement was necessary as our laboratory-made preparative chromatograph was not fitted with a detector. Fractions were collected as stated above and each was then examined for its contents on an analytical gas chromatograph.

In attempts to trap the effluent from the chromatograph quantitatively, a series of Drechsel bottles fitted with sintered glass distributors and containing acetone were connected in series with the outlet. Acetone was used as the solvent since it dissolved both the esters and the small amount of polyester "bleed" from the column. Separation of the fatty acid ester from the polyester can be effected by dissolution in petroleum ether, in which the polyester is insoluble. A series of three such bottles appeared to be an effective scrubber. It was decided to construct a vessel which would have the effective scrubbing properties of the three Drechsel bottles and which could be easily emptied without removal from the outlet of the chromatograph.

* The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research.

Construction of the trap

This is best described by reference to Fig. 1.

The trap consists of a central tube (E) fitted with three sealed-in sintered glass discs (F) of porosity No. 1 and with two side limbs (D) and (G). A water condenser (A) is fitted at the B 14 joint at the top of E. The limb D has a B 14 socket which carries a stoppered dropping funnel (B) and also a short side arm which connects the trap through the ball and socket union (C) to the heated outlet of the chromatograph. A short extension of the outlet internal to the ball and socket was found to reduce losses and serves to prevent intercontamination of fractions due to condensation at this point. The outlet (H) on the limb (G) is closed by a sealed tube carrying a B 10 socket which fits over the outlet and is held secure by a spring attached to lugs on both parts.

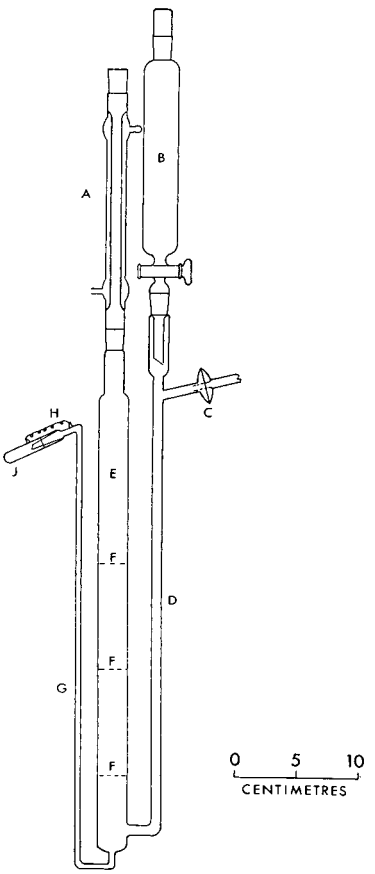

Fig. 1. Diagram of G.L.C. collection trap.

Operation of the trap

With the sealing tube (J) in place, the dropping funnel (B) is filled with acetone. The tap is opened and by applying pressure by means of a rubber blow-ball fitted with a B 14 cone to the top of the funnel, solvent is forced into the main chamber.

This is continued until there is a layer of solvent about one inch deep above the upper sintered disc and rather deeper layers on the other two. The tap is closed and the funnel stoppered. The trap is now ready for scrubbing the effluent gas. In order to cleanse any condensate from the limb (D), a slow solvent drip from the funnel may be used or alternatively a quick wash from the tap-funnel may be given before changing fractions. To change fractions the procedure is as follows: The tap-funnel (B) is closed and lifted from its socket. This allows the sealing tube (J) to be removed without discharging the trap. On replacing the tap-funnel the solution in that part of the trap under the lower disc is discharged through tube H into the collecting vessel by the gas flow from the chromatograph. The solvent above the discs is then forced into the lower part of the trap and expelled through tube H by gentle pumping with a rubber blow-ball at the top of the condenser. If it is so desired, a little wash solvent can be added at the top of the condenser and then pumped through. The seal (J) is then replaced and the trap re-filled with solvent as before. The procedure, which in description is rather complex, is in practice very simple and a change of fraction can be performed in less than one minute. Consideration has been given to the possibility of sophistication of the trap, for example by the incorporation of various taps to make discharge automatic but it was felt that this would probably be obtained only at the expense of increased fragility, at risk of contamination, and with little or no gain in efficacy.

On test runs with samples of fatty acid methyl esters, recoveries have been better than 95 %. The trap is easily constructed by a glass-blower, all parts being readily available. (The main chamber of our original trap was made by joining three sintered glass Gooch crucibles together.) It appears to have certain advantages over the traps hitherto described in that it is suited to the collection of a large series of fractions and requires no electrical connections, sources of heat or refrigeration.

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Dünnschichtchromatographische Abtrennung von γ-Aminobuttersäure aus Hirnextrakten

Im Stoffwechsel der freien Aminosäuren des Gehirns spielt die γ-Aminobuttersäure neben der Glutaminsäure und deren Halbamid, dem Glutamin, eine entscheidende Rolle. Da für Glutaminsäure und Glutamin einfache Bestimmungsmethoden existieren (enzymatische Bestimmung der Glutaminsäure mit Glutamatdehydrogenase[1] bzw. Hydrolyse des Amidstickstoffs des Glutamins[2]), andererseits die spezifische enzymatische Bestimmung der γ-Aminobuttersäure[3–7] wegen der schwierigen Enzympräparation sehr aufwendig ist, wurde eine einfache chromatographische Methode zur Abtrennung der γ-Aminobuttersäure ausgearbeitet.

Die aus der Literatur bekannten papierchromatographischen zweidimensionalen Verfahren zur Abtrennung aus Hirnextrakten[8,9] sind ebenso wie die säulenchromatographischen Verfahren[10,11] sehr zeitaufwendig, die eindimensionalen papierchromatographischen Methoden[12–14] lassen sich nicht ohne weiteres auf Dünnschichtplatten übertragen.

Die üblichen bekannten eindimensionalen[15–17] und zweidimensionalen[18–20] dünnschichtchromatographischen Verfahren enthalten keine Angaben über γ-Aminobuttersäure[15–19] und erwiesen sich für die Abtrennung aus Hirnextrakten wenig geeignet.

Aus den angeführten Gründen wurde ein neues dünnschichtchromatographisches Trennverfahren entwickelt, wobei der eindimensionalen Methode wegen besserer Reproduzierbarkeit sowie Zeit- und Materialersparnis der Vorzug gegeben wurde.

Material und Methoden

(1) *Extraktbereitung.* Mit flüssiger Luft eingefrorenes Gehirn äthernarkotisierter Albinoratten wurde mit 10 Teilen (Gew./Vol.) 80 %igem Äthanol homogenisiert, der Überstand nach Zentrifugieren in der Kälte 2 St. bei —15° gehalten und die ausgefallenen Lipide erneut abzentrifugiert. Dieser Extrakt wurde direkt zur Bestimmung verwendet (beim Eindampfen im Wasserbad bis zur Trockne und anschliessendem Aufnehmen mit Wasser oder Alkohol[8] treten Verluste von 12 bis 15 % auf!).

(2) *Bereitung der Platten und Aufbringen der Proben.* Glasplatten 20 × 20 cm und 10 × 20 cm wurden in üblicher Weise mit Kieselgel D (VEB Chemiewerk Greiz-Dölau*) in ca. 250 μ dicker Schicht beschichtet und nach dem Trocknen in 1 cm breite Bahnen mittels eines spitz ausgezogenen Glasstabes entlang der Laufrichtung eingeteilt. Am Startpunkt, 1.5 cm vom unteren Ende, wurden auf jede Bahn 20 oder 30 μl des alkoholischen Extraktes in Portionen von ca 1 μl aufgebracht, ferner auf zwei Bahnen jeder Platte 0.01 μMol. eines γ-Aminobuttersäurestandards (10 μl). Die Entwicklung erfolgte aufsteigend bei einer Gesamtlaufstrecke von 10 cm und einer Laufzeit von 1.5–2.5 St.

(3) *Quantitative Auswertung.* Zur quantitativen Auswertung wurde das Verfahren von BARROLLIER *et al.*[21,22] benutzt. Die Platten wurden nach Chromatographie mit dem Cadmiumacetat–Ninhydrin-Reagenz besprüht und 20 Min. bei 105° getrocknet (bei 20 Min. liegt das Maximum der Farbentwicklung). Die angefärbten

* Kieselgel D (VEB Chemiewerk Greiz-Dölau) enthält 13 % Gips und ist bei der Trennung von Aminosäuren dem Kieselgel G (Merck) gleichwertig.

Flecken wurden mit 30–50 μl einer 2 %igen Collodium-Lösung bedeckt, der nach dem Abdunsten des Lösungsmittels sich von der Platte abhebende Film mit einer Nadel in ein Zentrifugenglas überführt und mit 1 ml Cadmiumacetat-haltigem Methanol unter mehrmaligem Rühren extrahiert. Nach 30 Min. wurde das Kieselgel abzentrifugiert und der Überstand bei 1 cm Schichtdicke im monochromatischen Licht bei 500 nm gemessen. Die Extinktionen steigen laufend an, vermutlich durch den Ammoniakgehalt der Luft, jedoch beträgt die Extinktionszunahme im Zeitraum von 1–2 St. nach Beginn der Extraktion weniger als 3 %.

Obwohl die Ablösung des Farbstoffs vom Kieselgel nicht quantitativ ist, wird das LAMBERT-BEERsche Gesetz bis zu 0.1 μMol. γ-Aminobuttersäure streng erfüllt.

Bei Verwendung von Ammoniak-haltigen Laufmitteln müssen die Platten vor der Anfärbung 2 St. bei mindestens 105° ausgeheizt werden.

Ergebnisse

Folgende Lösungsmittelsysteme wurden zur eindimensionalen Abtrennung der γ-Aminobuttersäure aus Hirnextrakten untersucht. (Mischungverhältnisse in Volumenanteilen, Nr. 1 und 2 in Gewichtsanteilen.)

1. Phenol–Wasser (3:1).
2. *n*-Butanol–Eisessig–Wasser (6:2:2).
3. Methanol–Chloroform–Wasser (2:2:1).
4. Methanol–Chloroform–17 % Ammoniak (2:2:1).
5. Methanol–Chloroform–25 % Ammoniak (2:2:1).
6. 96 % Äthanol–Wasser (7:3).
7. 96 % Äthanol–25 % Ammoniak (7:3).
8. *n*-Propanol–Wasser (7:3).
9. *n*-Propanol–25 % Ammoniak (7:3).
10. Isopropanol–Wasser (7:3).
11. Isopropanol–5 % Ammoniak (7:3).
12. Isopropanol–10 % Ammoniak (7:3).
13. Isopropanol–17 % Ammoniak (7:3).
14. Isopropanol–25 % Ammoniak (7:3).
15. Isopropanol–35 % Ammoniak (7:3).
16. Collidin, wassergesättigt.

Von den untersuchten Laufmitteln erwiesen sich Nr. 6, 8, 10, 11 und 12 geeignet. In diesen Systemen wandert die γ-Aminobuttersäure am langsamsten. System Nr. 15 kann ausserdem zur Abtrennung von Glutaminsäure, Glutamin, Asparaginsäure und γ-Aminobuttersäure aus Hirnextrakten verwendet werden. Die R_F-Werte der brauchbaren Lösungsmittelsysteme sind für die wichtigsten freien Aminosäuren des Gehirns in Tabelle I dargestellt. Das stets der Front am nächsten laufende Taurin kann mit allen Systemen gut abgetrennt werden. Von den Alkohol–Wasser-Gemischen ergibt das System Nr. 10 die am schärfsten begrenzten Flecke.

Die eindimensionale Abtrennung der γ-Aminobuttersäure an gepufferten Kieselgel-Platten[16] (0.1 *M* Phosphatpuffer, pH 7.0, 9.7, 10.2 und 12.0) gelang in den Lösungsmitteln Nr. 6, 8 und 10 nicht, ebenso versagte in den gleichen Laufmittelsystemen und in Nr. 1 die Abtrennung über die Kupferkomplexbildung der α-Aminosäuren[23] durch Aufgeben von basischem Kupfercarbonat, einer Suspension von

TABELLE I

R_F-WERTE DER WICHTIGSTEN FREIEN AMINOSÄUREN DES GEHIRNS IN DEN ZUR ABTRENNUNG DER γ-AMINOBUTTERSÄURE GEEIGNETEN LAUFMITTELN

Aminosäure	*R_F-Wert* in Laufmittel Nr.*					
	6	*8*	*10*	*11*	*12*	*15*
Glycin	0.43	0.33	0.50	0.32	0.31	0.29
Alanin	0.46	0.40	0.52	0.38	0.41	0.39
Asparaginsäure	0.49	0.40	0.52	0.32	0.28	0.11
Glutaminsäure	0.48	0.40	0.48	0.40	0.34	0.15
Glutamin	0.43	0.37	0.48	0.48	0.46	0.37
γ-Aminobuttersäure	0.34	0.27	0.35	0.22	0.18	0.23
Taurin	0.59	0.53	0.62	0.52	0.51	0.48

* Mittelwerte aus mindestens 4 Einzelbestimmungen.

basischem Kupfercarbonat oder Auftropfen einer Kupfersulfatlösung am Startpunkt vor Aufgabe der zu trennenden Substanzen. Bei 17 Paralleluntersuchungen eines Extraktes wurde eine Streuung von $\pm 12.6\,\%$ ermittelt (Laufmittel Nr. 10).

Der Gehalt an γ-Aminobuttersäure im Hirn äthernarkotisierter Albinoratten beträgt 2.58 ± 0.29 μMol./g Hirn-Frischgewicht (N = 13).

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* Direktor: Prof. Dr. R. BAUMANN.

Two methods for the thin-layer chromatographic separation of terpene alcohols and identification by a colour reaction

Method 1

Following a suggestion of Dr. B. H. DAVIES (Department of Agricultural Biochemistry, University College of Wales), a reversed-phase TLC separation of C_{10}, C_{15} and C_{20} terpene alcohols has been achieved on paraffin-impregnated Kieselgur G using paraffin-saturated aqueous acetone as the mobile phase. The result is shown in Fig. 1, in which it can be seen that the R_F value decreases with increasing chain length and that the positional isomer travels behind the corresponding primary alcohol. The component with the highest R_F value in the mixture was an impurity present in some of the individual alcohols.

Procedure

Kieselgur G layers (0.25 mm thick) were prepared using the Desaga spreader and the plates were dipped once into a 5 % solution of liquid paraffin in petroleum ether (b.p. 40–60°). After the solvent had evaporated, the alcohols (0.1 % solution in benzene) were applied (1–10 μl per spot). A mixture of acetone (65 ml) water (35 ml) and liquid paraffin (0.5 ml) was shaken and the lower paraffin-saturated layer was used in a closed tank lined with filter paper. After the solvent had risen to the desired level, the plate was removed from the tank and allowed to dry on the bench, during which time the alcohols were visible as white spots on the damp background. The linalool spot was thus seen just behind that of geraniol, although it was indistinct after spraying. They could be located with iodine vapour, a more permanent result

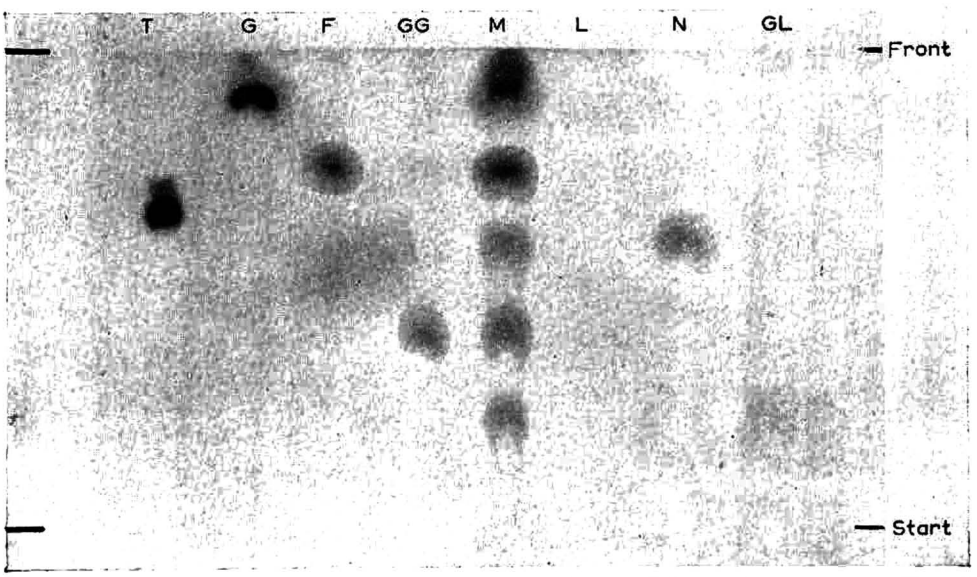

Fig. 1. Terpene alcohols on paraffin-impregnated Kieselgur G. Solvent: Acetone–water (65:35) saturated with paraffin. Distance travelled: 15 cm (90 min). Spray: Anisaldehyde (0.5 g), conc. sulphuric acid (0.5 ml) and 90% ethanol (9 ml). Test mixture (T): Butter Yellow, Sudan Red G, indophenol blue. (M): Synthetic mixture of terpene alcohols.

being obtained with anisaldehyde–sulphuric acid spray. The plate was photographed for reproduction but an excellent record was also obtainable by the use of the Dalcopier reflex photocopier. In the latter case the layer was covered with cellophane, which was secured with Cellotape to the underside of the glass, and a photograph taken by reflected light.

Method 2 and colour reaction

TYIHAK, VAGUJFALVI AND HAGONY[1] reported the separation of *trans-trans*- and *cis-trans*-farnesol on silica gel, using benzene–ethyl acetate (95:5) in ascending chromatography. This system, slightly modified, gives a good separation of all the stereoisomers of farnesol present in a commercial mixture and it is now shown to separate the C_{10}, C_{15} and C_{20} terpene alcohols as well as the positional isomers. In contrast to method 1, the R_F value of the alcohol is greater, the longer the carbon chain; a still larger effect is produced by a change in position of the hydroxyl group. Fig. 2 shows this, where, for example, the difference between geraniol and linalool is greater than that between geraniol and farnesol. Although the separation may not

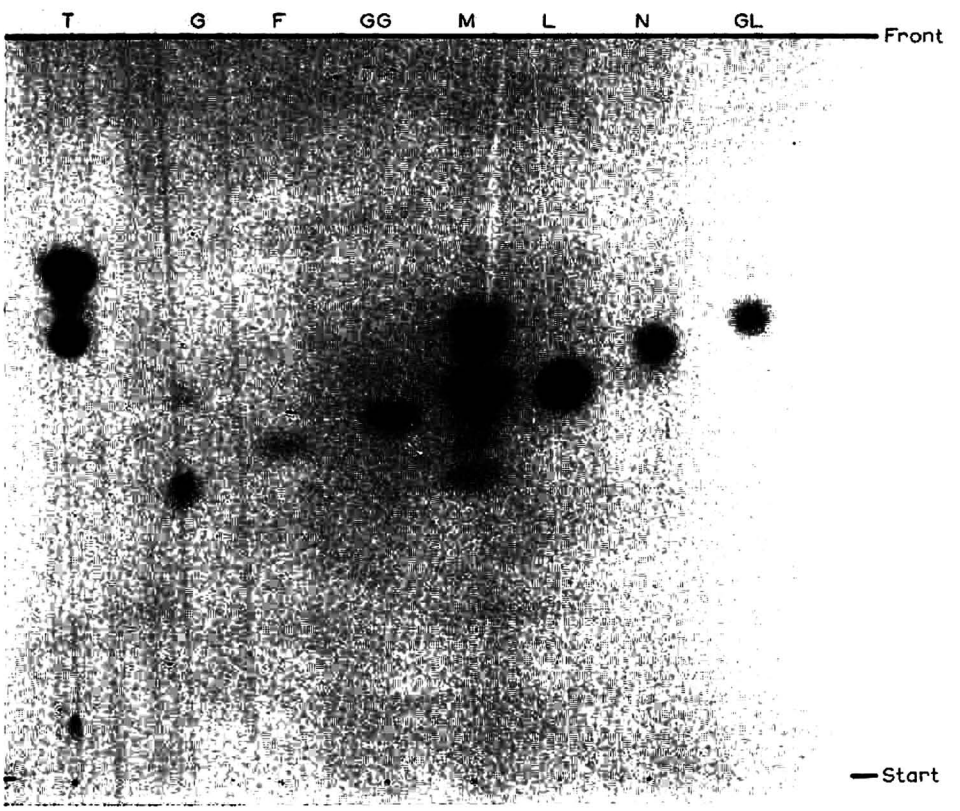

Fig. 2. Terpene alcohols on Kieselgel G (0.25 mm thick), activated 30 min at 110°. Solvent: Benzene–ethyl acetate (80:20). Distance travelled: 15 cm (45 min). Spray: Anisaldehyde–H_2SO_4 (as in Fig. 1) after exposure to iodine vapour.

be clear on the reproduction, additional identification is provided by a colour reaction shown by these alcohols. Preliminary location by exposure to iodine vapour gives the usual yellow spots. If most of the iodine be allowed to evaporate and the plate sprayed with anisaldehyde–sulphuric acid, characteristic colours are produced on gentle heating. These are shown in Table I, together with the R_R values, the distance travelled compared with that by Sudan Red, a component of the Desaga test mixture.

TABLE I

R_R* VALUES OF TERPENE ALCOHOLS AND COLOURS AFTER SPRAYING

Compound	R_R	*Colour***
Geraniol	0.60	Dark blue
trans-trans-Farnesol	0.69	Purple
Geranyl geraniol	0.75	Mauve
Linalool	0.85	Green
Nerolidol	0.95	Brownish yellow
Geranyl linalool	1.01	Yellowish brown

* Distance travelled compared with Sudan Red.
** After exposure to iodine and spraying with anisaldehyde–H_2SO_4.

These colours fade within 2 h; after this time, further heating produces the usual sulphuric acid charring. The differential colour reaction is not shown in the reversed-phase system.

Materials

Geraniol (G) and farnesol (F) were commercial materials (Koch-Light Laboratories Ltd.). Geranyl geraniol (GG) and nerolidol (N) were kindly supplied by Dr. B. H. DAVIES and geranyl linalool (GL) by Dr. E. DEMOLE. Kieselgel G and Kieselgur G were commercial products (E. Merck).

For method 2, the farnesol and geranyl geraniol were purified by preliminary preparative-scale TLC on Kieselgel G, using benzene–ethyl acetate (80:20). The bands were located by spraying sample side strips with anisaldehyde reagent, the main area of the plate being covered by a sheet of glass. In each case, the slower of the two main bands was scraped off and extracted with ethanol. That of farnesol was the *trans-trans*-isomer.

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1 E. TYIHAK, D. VAGUJFALVI AND P. L. HAGONY, *J. Chromatog.*, 11 (1963) 45.

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Zur Berechnung optimaler Trennungsbedingungen für zwei Stoffe mit bekannten R_F-Werten bei mehrfach wiederholter Papierchromatographie

Bei der papierchromatographischen (bzw. Dünnschicht-) Trennung von zwei Stoffen mit wenig unterschiedlichen R_F-Werten ist es häufig von Vorteil, ein Lösungsmittel-System, in dem beide Stoffe relativ kleine R_F-Werte besitzen, anzuwenden, und das Lösungsmittelgemisch mehrere Male über die Substanzen auf- oder absteigen zu lassen[1]: auf diese Weise steigt die Zahl der Verteilungsvorgänge.

Für die Ermittlung günstiger Trennungsbedingungen haben LENK[2] ein Diagramm und THOMA[5] Tabellen angegeben. Die Zahl der "Läufe", die zu einer optimalen Trennung führt, lässt sich jedoch einfacher allgemein berechnen, wenn man die R_F-Werte beider Stoffe kennt. Ein Stoff A mit dem R_F-Wert a hat nach n Läufen die relative Strecke:

$$S_a = 1 - (1 - a)^n \qquad (1)$$

zurückgelegt. Soll der Stoff A vom Stoff B mit dem R_F-Wert b getrennt werden, so sind beide nach n Läufen um:

$$D = S_a - S_b = (1 - b)^n - (1 - a)^n$$

auseinandergewandert. Bei $dD/dn = 0$ erreicht D ein Maximum; n ist hier optimal. Die Differentiation der Funktion:

$$D = (1 - b)^n - (1 - a)^n = e^{n \cdot \log(1 - b)} - e^{n \cdot \log(1 - a)}$$

ergibt:

$$\frac{dD}{dn} = e^{n \cdot \log(1 - b)} \cdot \log(1 - b) - e^{n \cdot \log(1 - a)} \cdot \log(1 - a)$$

$$n_{opt.} = \frac{\log \dfrac{\log (1 - a)}{\log (1 - b)}}{\log \dfrac{(1 - b)}{(1 - a)}} \qquad (2)$$

Als Beispiel sei nachstehend die Trennung der Zucker D-Glucose und D-Galactose angeführt. Beide Zucker wurden nebeneinander streifenförmig aufgetragen und nach zwölfstündigem Äquilibrieren im Chromatographiegefäss mit *n*-Butylacetat–Essigsäure–*n*-Butanol–Methanol–Wasser (3:2:2:1:1)[4] absteigend chromatographiert. Nach jedem Lauf wurde ein Streifen des Chromatogramms abgeschnitten und mit $AgNO_3$–NaOH[5] entwickelt. Fig. 1 zeigt einen Vergleich der experimentell ermittelten und der nach Gl. (1) berechneten Laufstrecken.

Die relativ geringen Abweichungen der experimentell ermittelten Laufstrecken von den berechneten sind durch Temperaturschwankungen und zunehmende chemische und mechanische Beanspruchung des Chromatographiepapiers bedingt. Sie machen sich bei der Bildung der Differenz naturgemäss stärker bemerkbar. Die berechnete Funktion der Laufstreckendifferenzen besitzt nach Gl. (2) bei 8 Läufen

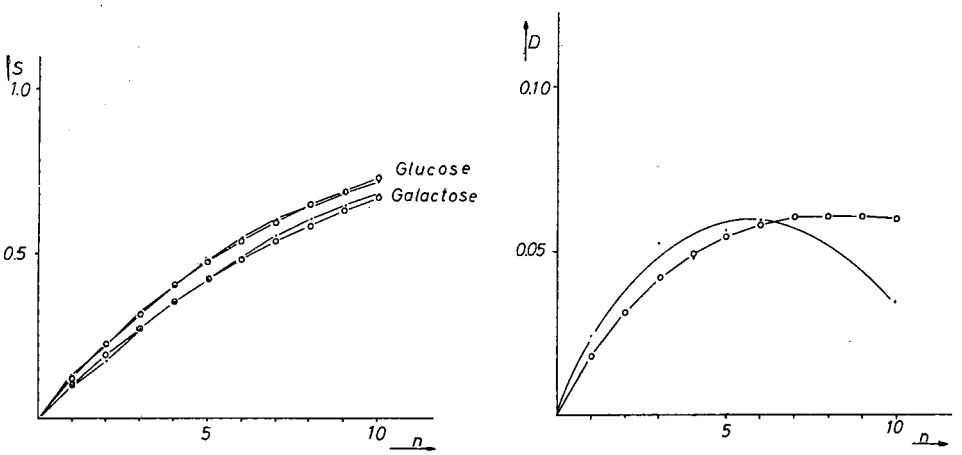

Fig. 1. Vergleich der berechneten und der gefundenen Wanderungsstrecken für die papierchromatographische Trennung von Glucose und Galactose. Abszisse: Zahl der chromatographischen Läufe des Lösungsmittels (n); Ordinate: relative Wanderungsstrecken (S). —o—o— berechnete, —·—·— gefundene Werte.

Fig. 2. Vergleich der berechneten und der gefundenen Differenzen der Wanderungsstrecken von Glucose und Galactose. Abszisse: Zahl der chromatographischen Läufe des Lösungsmittels (n); Ordinate: Differenzen der Wanderungsstrecken (D). —o—o— berechnete, —·—·— gefundene Werte.

ein sehr flaches Maximum, der experimentell bestimmte Wert liegt mit 6 Läufen etwas darunter (Fig. 2). Trotzdem dürfte die Gl. (2) ein nützliches Hilfsmittel sein, um die optimalen Trennungsbedingungen abzuschätzen.

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Paper chromatography of lipids: use of cupric hydroxide impregnated paper

A method of paper chromatography was developed using cupric hydroxide impregnated paper for the analysis of lipids.

Whatman 3 MM paper was impregnated by immersion, first in 5 % cupric sulphate solution and then, after drying, in a 5 % sodium hydroxide solution. The paper was then washed with tap water until no basic reaction was observed anymore, and finally with distilled water. After drying (at a temperature not exceeding 80°), the prepared paper may be stored for an indefinitive time, but away from acid vapours.

The following ^{14}C-labelled fatty acids and glycerides were examined with this paper: oleic, linoleic, linolenic, palmitic, stearic, myristic and lauric acids, diolein and triolein.

A sample (25–50 μg), dissolved in chloroform, was spotted on the paper and developed by ascending chromatography using the solvents shown in Table I. After development, the chromatograms were scanned with a thin-window Geiger-Müller counter to determine the corresponding peaks of radioactivity (see Table I).

TABLE I

R_F VALUES

	*Solvents**			
	1	*2*	*3*	*4*
Oleic acid	0.68	0.99	0.72	0.02
Linoleic acid	0.54	0.98	0.74	0.04
Linolenic acid	0.80	0.96	0.80	0.09
Triolein	0.00	0.99	0.00	0.98
Diolein	0.00	0.96	0.02	0.94
Stearic acid	—	0.00	0.72	0.04
Palmitic acid	—	0.00	0.68	0.50
Myristic acid	Do not form copper soaps			
Lauric acid	Do not form copper soaps			

* Solvent 1: 10% cyclohexylamine in water.
Solvent 2: Chloroform–ethanol (1:1).
Solvent 3: Methanol–water–cyclohexylamine (120:60:10).
Solvent 4: Benzene–chloroform (1:2).

The spots were also detected, after a short treatment with 0.1 % acetic acid to eliminate the excess of cupric hydroxide from the paper, by staining according to KAUFMANN AND NITSCH[1] with potassium ferrocyanide.

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1 H. P. KAUFMAN AND W. H. NITSCH, *Fette und Seifen*, 56 (1954) 154.

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Reinigung synthetischer Peptide mit der trägerfreien präparativen Durchfluss-Elektrophorese

Neben einer analytischen elektrophoretischen Auftrennung von polaren Substanzgemischen haben in den letzten Jahren die verschiedensten präparativen Techniken praktische Bedeutung erlangt. Im wesentlichen wird nach zwei prinzipiell unterschiedlichen Methoden gearbeitet: der diskontinuierlichen Zonen-Elektrophorese und der kontinuierlichen Durchfluss-Elektrophorese. Bei beiden Verfahren werden Trägersubstanzen wie Papierblätter, Papierkarton, Glaspulver, Stärkegel, Cellulose, Sephadex® etc. verwendet. Eine wesentliche Verbesserung für präparative Trennungen bietet das Prinzip der trägerfreien präparativen Durchfluss-Elektrophorese wie sie von BARROLLIER *et al.*[1] und HANNIG[2] beschrieben wurde. Das Problem einer exakten Temperaturregelung wurde durch Wasserkühlung[1*], bzw. Luftkühlung[2,**] gelöst. Über eine Verwendung des von BARROLLIER *et al.*[1] entwickelten und in der Zwischenzeit verbesserten Gerätes (vgl. Zit. 3-6), zur Trennung von Serumeiweiss ist mehrfach berichtet worden[4,5,7].

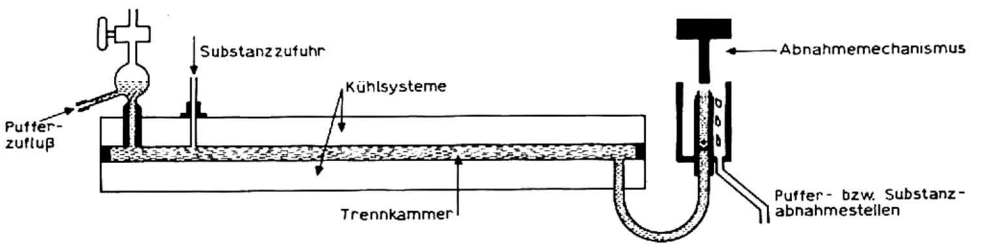

Fig. 1. Schematische Skizze des "Pheroplan". Die auf der oberen Kühlplatte parallel zum Pufferstrom angeordneten Elektrodentröge mit in die Trennkammer ragenden Diaphragmen sind nicht eingezeichnet.

Seit längerer Zeit hat sich die trägerfreie Durchfluss-Elektrophorese ("Pheroplan" ®, vgl. schematische Skizze, Fig. 1) in unserem Laboratorium auch zur kontinuierlichen präparativen Reinigung von synthetischen Peptiden hervorragend bewährt. Die Erfahrungen, die besonders an Peptiden von Angiotensin-[8,9], Bradykinin-[10–14] und Kallidin-Typ[15], sowie auch an anderen basischen und neutralen Peptiden gesammelt wurden, sollen im folgenden an einigen Beispielen demonstriert werden.

Bedingungen für präparative Trennung eines Peptidgemisches

(1) Flüchtiger Puffer für die Trennkammer: z.B. 0.033 *M* Pyridiniumacetat, pH 5 (~ 600 Ω/cm).

(2) Puffer für die Elektrodentrogspülung: 0.1 *M* Pyridiniumacetat, pH 5 (die höhere Konzentration verhindert Ionenverarmung und pH-Wert Änderungen). Die Spülung (~ 1–2 l/Std.) erfolgt in der Weise, dass der ablaufende Spülpuffer keine wesentliche Änderung des pH-Wertes und der Leitfähigkeit zeigt.

* "Pheroplan" ® der Firma K. Marggraf o.H.G., Grolmannstr. 44–45, Berlin-Charlottenburg.
** "Elphor VAP Apparatur" ® für Trennungen im reinen Pufferfilm, Firma Dr. Bender und Dr. Hobein, Lindwurmstr. 71–73, München.

(3) Kühlung: der Kühlsoleumlauf wird so eingestellt, dass die Sole nach Passieren des Pheroplan-Kühlsystems $\sim 0°$ bis $+1°$ hat.

(4) Spannung: 2000–3000 V.

(5) Stromstärke: 120–190 mA.

(6) Konzentration der zu trennenden Substanz: 1 ml/Std. einer 5%igen Lösung des Peptidgemisches.

(7) Verweilzeit: die von dem Flüssigkeitsstand in den Überlaufgefässen und der Pufferabnahme abhängige Zeit, in der die Substanz in der Trennkammer unter Spannung verbleibt, beträgt 60–90 Min. Bei zu hoher Verweilzeit kann eine Wanderung der Substanz in die Elektrodentröge erfolgen.

(8) Dosierstelle (Einpumpstutzen für die Substanz): erste Stelle ab Anode zwecks Erzielung einer möglichst grossen Trennstrecke.

Die angegebenen Daten stellen Standardbedingungen dar. Abhängig von der Ladung des Peptids sowie der Lage der Verunreinigungen im Vergleich zur Hauptzone (analytisches Elektrophoresebild) können die Trennbedingungen variiert werden. Für eine optimale Trennung eines Peptidgemisches und eine gute Ausnutzung der Trennmöglichkeit mit dem Pheroplan muss gegebenenfalls ein Vorversuch (50 mg Substanz) durchgeführt werden. Für die angeführten Peptide erwies sich die Verwendung des Pyridiniumacetatpuffers pH 5 als besonders geeignet. In einigen Fällen wurden auch mit Essigsäure–Ameisensäure-Puffer pH 1.7 gute Trennergebnisse erzielt.

Die Fig. 2–5 demonstrieren die äusserst scharfen Trennungen der Hauptkomponenten von geringer bzw. stärker basischen Begleitpeptiden, die am Anfang bzw. Ende der aufgefangenen Fraktionen konzentriert sind (z.B. Abb. 2: Fraktion 32–38 reine Hauptfraktion; Fraktion 19–31 Hauptfraktion + schwächer basische Verunreinigung; Fraktion 39–48 stärker basische Verunreinigungen. Die am Startpunkt eingekreisten Markierungen sind nicht wandernde Farbstofflecke zur Kenn-

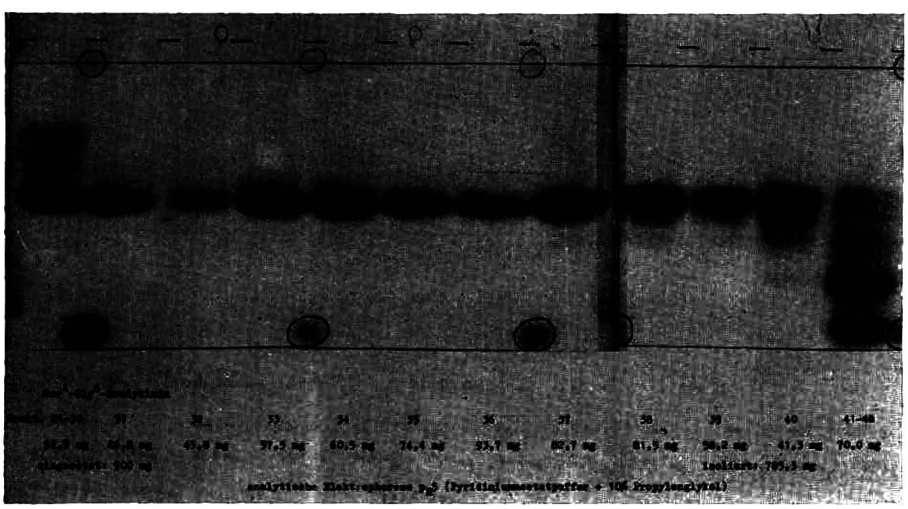

Fig. 2. "Pheroplan-gereinigtes" Ser^4-Gly^6-Bradykinin. Analytische Papierelektrophorese der einzelnen isolierten Fraktionen in Pyridiniumacetatpuffer pH 5 + 10% Propylenglycol (10 V/cm, 6 Std., Ninhydrinfärbung).

zeichnung der Endosmose, bei den unteren eingekreisten Flecken handelt es sich um Ornithin als Bezugssubstanz). Selbst im analytischen Bild sehr dicht neben der Hauptkomponente liegende Verunreinigungen lassen sich glatt abtrennen. Von den zur Reinigung eingesetzten Substanzmengen werden im Durchschnitt 85–95 % nach der Trennung wieder isoliert. Im kontinuierlichen Durchsatz konnten Peptide in Mengen bis zu 4–5 g getrennt werden. Die elektrophoretische Reinigungsmethode

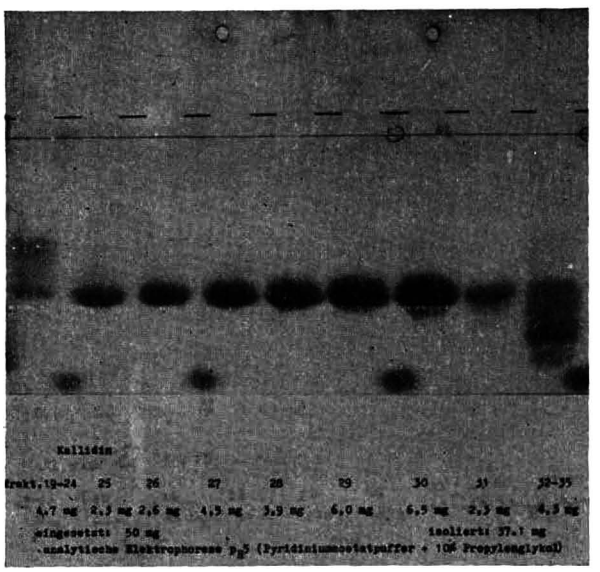

Fig. 3. "Pheroplan-gereinigtes" Kallidin. Analytische Papierelektrophorese.

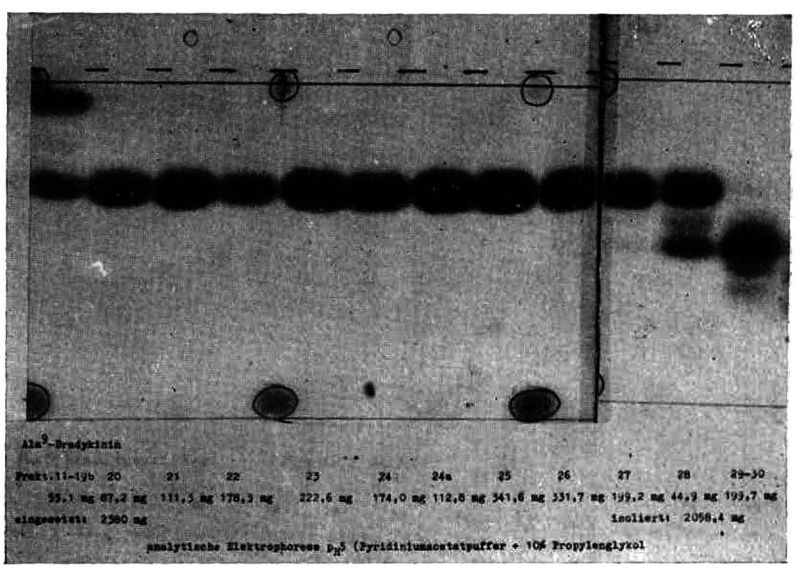

Fig. 4. "Pheroplan-gereinigtes" Ala⁹-Bradykinin. Analytische Papierelektrophorese.

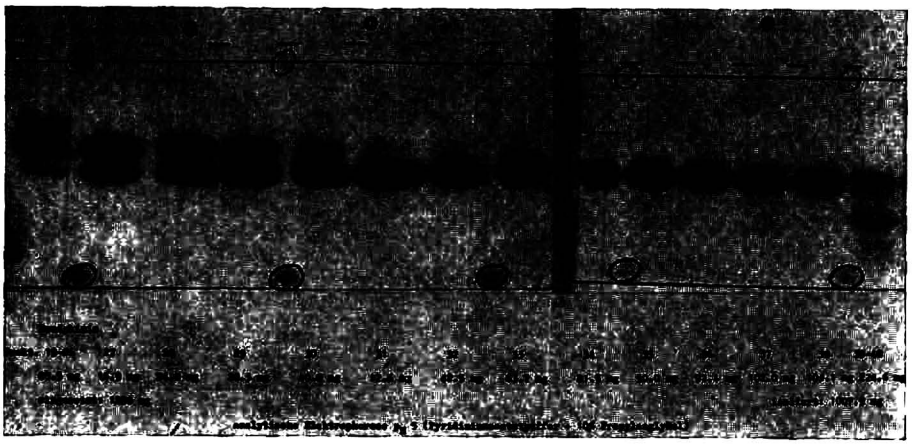

Fig. 5. "Pheroplan-gereinigtes" Bradykinin. Analytische Papierelektrophorese.

bietet eine wertvolle Ergänzung zur Säulenchromatographie an Austauscher-Cellulosen. In Fällen, wo selbst durch Variation der Gradienten-Elution (Konzentrations- bzw. pH-Änderungen) keine vollständige Reinigung gelang, konnte direkt durch die trägerfreie Durchfluss-Elektrophorese bzw. durch Elektrophorese im Anschluss an eine Säulenvorreinigung eine Reinigung erzielt werden. Für kleine Substanzmengen (100–500 mg) d.h. Mengen, in denen hoch wirksame biologisch aktive Peptide im allgemeinen zunächst synthetisiert werden, ist die präparative Elektrophorese auch wegen eines geringeren Zeitaufwandes der Säulentrennung vorzuziehen.

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Für wertvolle Diskussionen sind wir Herrn Dr. J. BARROLLIER, für eine Unterstützung bei den präparativen Versuchen Frl. RENATE GERICKE zu Dank verpflichtet.

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Counter current distribution of alkaloids with a pH gradient

This paper reports preliminary counter current distribution (C.C.D.) experiments, performed with a non-polar mobile phase and an aqueous stationary phase whose pH changes regularly from tube to tube.

The results show that substances whose ionization changes with pH can be separated in short runs if some physicochemical requirements are fulfilled.

Experimental

A 25 tube apparatus, 11.5 cc per phase, was employed. As mobile phase we utilized benzene or petroleum ether because more polar phases such as butyl alcohol gave strong gradient distortion during the run.

The aqueous layer consisted of the BRITTON AND ROBINSON universal buffer[1,2] distributed from the alkaline (first tube) to the acid zone with a gradient of about 0.3 pH unit per tube.

The alkaloids were dissolved in the organic solvent at concentrations of the order of 1 mg/cc.

After the run, they were located by measuring the absorbance of the aqueous layer at 270 mμ.

Results

Fig. 1 shows the results of the fractionation of a mixture of brucine and hydrastine, and Fig. 2, the separation of hydrastine and caffeine performed under similar conditions except for the limits of pH. The experiments show the presence of two components but the resolution is poor.

The C.C.D. carried out with a mixture of caffeine and papaverine (Fig. 3) showed the existence of an optimal transference number. In Fig. 4 we show the separation of a more complex mixture, ecgonine, strychnine, papaverine and caffeine.

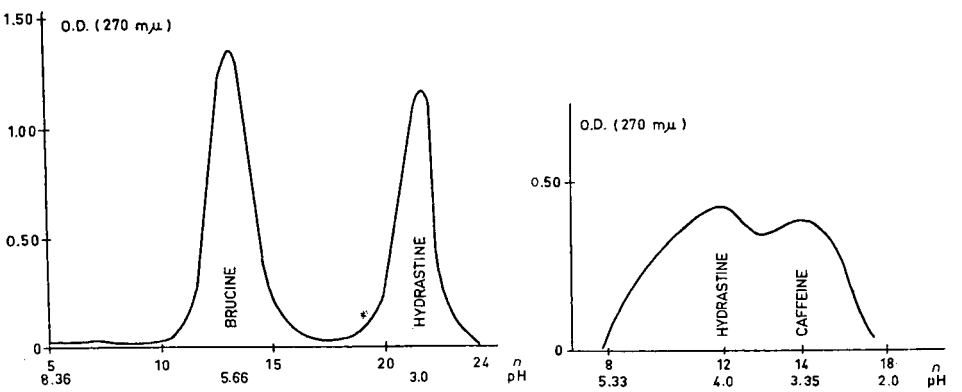

Fig. 1. C.C.D. of a mixture of brucine and hydrastine. Stationary phase BRITTON and ROBINSON universal buffer, pH range 9.0–2.5, 0.33 pH units per tube gradient. Mobile phase benzene. 25 transfers.

Fig. 2. C.C.D. of a mixture of hydrastine and caffeine.

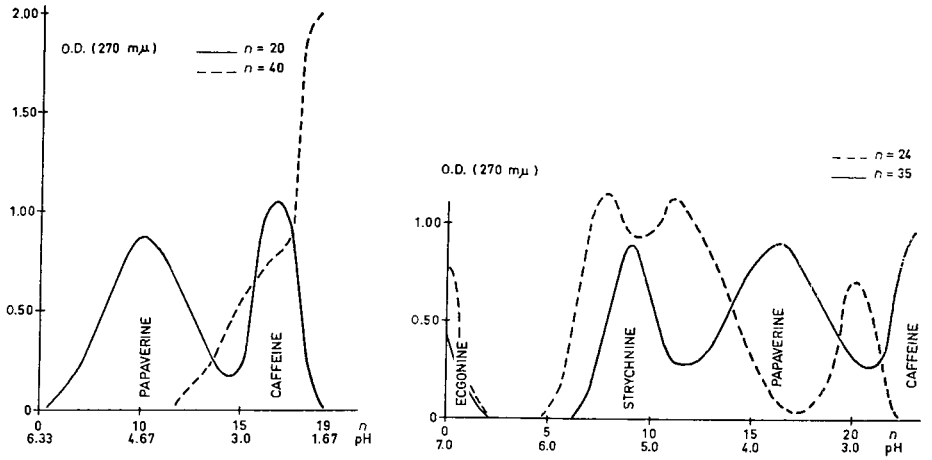

Fig. 3. Separation of the mixture papaverine and caffeine with BRITTON and ROBINSON universal buffer, pH range 8.0–2.0, 0.33 pH units per tube gradient. Mobile phase benzene. Full curve corresponds to the optimal number of transfers (20) and the dotted line to 40 transfers.

Fig. 4. Separation of a mixture of ecgonine, strychnine, papaverine and caffeine. pH range of the buffer 7.0–2.4, pH gradient 0.2 units per tube. Dotted line corresponds to 24 extractions and full line to 35 transfers.

Discussion

The separation of two alkaloids depends on their ionization and their extraction by the organic phase; the former is determined by the pK_b of the alkaloid and the latter by its partition coefficient.

When these properties serve to retard one of the substances with respect to the other, the separation is greatly facilitated. This occurs in the first example reported here, the constants for which are given in Table I.

TABLE I

IONIZATION CONSTANTS AND PARTITION COEFFICIENTS FOR VARIOUS ALKALOIDS

	pK_b	a^*
Brucine	6.04	1.86/0.1
Caffeine	14.2	0.36/1.35
Hydrastine	7.8	8.89/0.025
Papaverine	8.07	$\gg$0.36/1.35

* a is given as organic phase/aqueous phase; values taken from SEIDELL[3].

The ionization tends to retard brucine because it occurs at a higher pH value than for hydrastine; at the same time extraction due to benzene is greater for hydrastine. It is clear that in this system brucine will be the slower solute.

The situation in the second example (see Table I) is the opposite, and the separation is very difficult.

In the third experiment, papaverine ionizes first and during the twenty former

transfers caffeine moves forward. When both alkaloids are ionized, the situation is reversed because benzene extracts ionic papaverine more readily than it does caffeine, and consequently papaverine superimposes on caffeine.

Conclusion

In spite of some limitations, such as the inability of some substances to change their degree of ionization with a change of pH and restriction of the mobile phase to non-polar solvents, the method can be employed for the separation of appreciable amounts of weak acids or bases and ampholites in short runs. The C.C.D. results may be of use for batch extraction at controlled pH.

Brucine and hydrastine can, for instance, be separated almost quantitatively by a single extraction with benzene by adjusting the pH of the buffer to 4.0.

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J. Chromatog., 17 (1965) 193–195

Chromatographic estimation of asarones in Indian *Acorus calamus* Linn. oil (tetraploid variety)

The roots of *Acorus calamus* Linn., growing in the plains of India, have been used for the treatment of various ailments[1,2] from very ancient times. Pharmacological studies have shown that the essential oil and asarone (*trans* and *cis*) possess relaxant, spasmolytic[3,4], and hypotensive[5] properties and have powerful insecticidal activity[6]. The above properties have been shown to be due to the presence of asarones (*trans* and *cis* forms) which are present to the extent of 82 %[7] in the essential oil of the Indian *Acorus calamus* Linn. (tetraploid variety). It has been shown that asarones (asarone and β-asarone) are the important constituents of the oil which determine its quality. At present there is no method available for the estimation of asarones in the oil. It was, therefore, considered worthwhile to develop a method for the quantitative estimation of the asarone content of the Indian calamus oil, which is obtained from the roots of *Acorus calamus* (tetraploid variety with chromosome number $2n = 36$ ($x = 9$)).

Investigation of the oil showed that the hydrocarbon part could be separated easily by adsorbing it on a column of alumina and then eluting it with petroleum ether; the asarone part could then be eluted from the column with a mixture of benzene and ether (9:1). On the basis of the above observations the following method was, therefore, developed.

Method

1 g of the oil of *A. calamus* (tetraploid variety) was chromatographed over 50 g of grade I (Brockmann) alumina packed in a column of 2 cm diameter. It may be observed from the elution curve (Fig. 1) that 35 ml of petroleum ether (b.p. 40–60°) elutes all the hydrocarbons and then 65 ml of mixtures of benzene and ether (9:1)

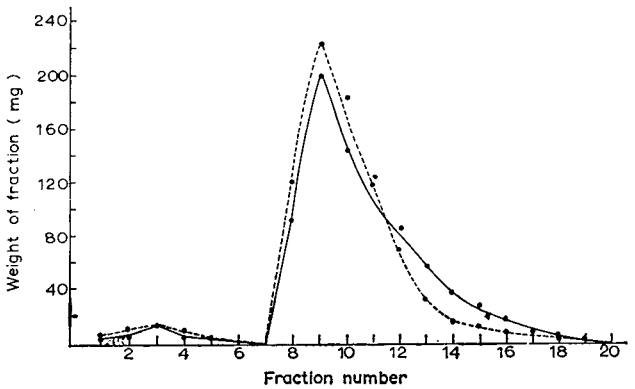

Fig. 1. Elution curve of asarone (*cis* and *trans*) from 1 g of *A. calamus* oil (tetraploid variety growing in the plains of India) over 50 g grade I alumina. Volume of each fraction: 5 ml. ——— oil distilled in 1963; – – – oil distilled in 1964.

elutes all the asarone present in the oil. The solvent is removed, the last traces being removed under vacuum and the residue weighed. The percentage of asarones can be calculated as follows:

$$\% \text{ Asarones} = \frac{\text{wt. of asarones}}{\text{wt. of oil}} \times 100$$

The method could not be verified by comparison with some conventional method. Nevertheless, we examined different samples of the oil distilled at various stages and found that the results were found to agree within 1 % (Fig. 1). The oils distilled in 1963 and 1964 gave 80.7 % and 79.9 %, respectively, as the asarone content.

The authors are grateful to Dr. I. C. CHOPRA, Director of this laboratory, for his keen interest in the above investigation.

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The radiochemical separation of some metals by partition chromatography with reversed phases on teflon in the system tri-*n*-octylamine–electrolyte

Partition chromatography in a liquid–liquid system, where one of the two phases is stationary and adsorbed on the inert, solid support as described by MARTIN AND SYNGE[1], finds more and more applications in the radiochemical analysis of inorganic compounds.

In studies on the conditions of separation of metal ions by partition chromatography with reversed phases the pulverised organic polymers are often used as the inert supports of the stationary phase. These include polytrifluorochloroethylene (KEL-F)[2–4], polytetrafluoroethylene (teflon)[5,6] and polyvinyl chloride with vinyl acetate[7]. Recently CERRAI AND TESTA[8] used tri-*n*-octylamine (TNOA) adsorbed on powdered cellulose for the separation of some metals, and TESTA[9] separated lanthanides by this method.

The reaction between the tertiary amines and metal ions $(MB^{m-}_{m+n})_{aq}$ can be described in the form of the equation characterizing the ion exchange:

$$(mR_3NH^+A^-)_o + (MB^{m-}_{m+n})_{aq} = (R_3NH)_m(MB_{m+n})_o + (mA^-)_{aq},$$

or as an addition reaction of the neutral molecule of the inorganic salt (MA_n):

$$(mR_3NH^+A^-)_o + (MA_n)_{aq} = (R_3NH)_mMA_{m+n_o},$$

where o indicates the organic phase and aq the aqueous phase.

According to the experimental data reviewed by COLEMAN[10], ISHIMORI AND NAKAMURA[11], the partition coefficients for the salts studied, when determined by the static extraction method using tri-iso-octylamine (TIOA) and hydrochloric acid differ according to the HCl concentration. Similarly in the case of TNOA, the choice of an appropriate concentration of HCl as eluant should afford the separation of some mixtures of metal halogenides, owing to the different values of their partition coefficients.

Experimental

A glass column (2.8 mm in diameter, 75–80 mm long) was used in the studies on the separation of mixtures of various metal chlorides. Powdered teflon $(-CF_2-CF_2-)_n$ (150–250 mesh) was introduced into the column as a suspension in benzene, washed with ethanol and then with water. About 375 mg TNOA (Fluka, Buchs S.G., Switzerland) was then adsorbed onto the teflon. After washing with water and 1 *N* HCl the column was ready for use. The radiotracer method was used in the investigations on metal separation. The experiments were carried out at room temperature (20° ± 2°). The flow rate of the eluants was regulated by means of a pressure of 0.3–0.5 kg/cm^2; one drop of eluate (0.03 ml) passed every 60 sec. The drops, collected on polystyrene foil, were dried with an infrared lamp. The measurements of the activity of the initial solutions and the eluates were performed with the aid of a Geiger-Müller end-window counter (AAH 55 type), while a flow counter 2π (AET-60 type) was used for measuring the activity of the ^{63}Ni isotope, and the activity of uranium was determined with a scintillation counter according to the method de-

TABLE I

CONCENTRATIONS OF METALS USED IN THE SEPARATIONS

Salt	*Concentration of metal ion (M)*	*Radio-indicator*	*Half-life*
$CaCl_2$	$4.5 \cdot 10^{-4}$	^{45}Ca	163.5 d
$MnCl_2$	$2.5 \cdot 10^{-6}$	^{54}Mn	291 d
$FeCl_3$	$1 \cdot 10^{-3}$	$^{55,59}Fe$	2.6 y 45.1 d
$CoCl_2$	$1.2 \cdot 10^{-6}$	^{60}Co	5.24 y
$NiCl_2$	$2.1 \cdot 10^{-3}$	^{63}Ni	125 ± 6 y
$CuCl_2$	$5 \cdot 10^{-4}$	^{64}Cu	12.9 h
$ZnCl_2$	$7 \cdot 10^{-3}$	^{65}Zn	244.3 d
$SrCl_2$	$1.3 \cdot 10^{-5}$	^{90}Sr	27.7 y
YCl_3	Carrier-free	^{90}Y	64.6 h
$CdCl_2$	$1.1 \cdot 10^{-4}$	^{115}Cd	42.6 d
$ThCl_4$	Carrier-free	^{234}Th	24.1 d
$PaCl_5$	Carrier-free*	^{233}Pa	27 d
UO_2Cl_2	$1.9 \cdot 10^{-2}$	Natural	

* In the presence of $ThCl_4$, conc. $1 \cdot 10^{-4}$ *M*.

scribed previously[12]. The concentrations of the chlorides of the separated metals, labelled with the radioactive isotopes, are given in Table I.

It was found during the course of the experiments that chlorides of iron, zinc, and cadmium are strongly adsorbed from 1 *N* HCl-solution, in contrast to chlorides of manganese, cobalt, copper, calcium, strontium, and yttrium, which are not adsorbed and are eluted in the first free volume of the column. The ferric chloride adsorbed was eluted with 0.01 *N* HCl, but zinc and cadmium chlorides formed stable complexes with TNOA which are not eluted with diluted HCl or HNO_3.

In order to elute zinc and cadmium, the column was washed with water till a neutral reaction was obtained and then eluted with the buffer 0.2 *M* ammonium acetate–ammonium hydroxide (pH = 10). The results obtained allowed the separation

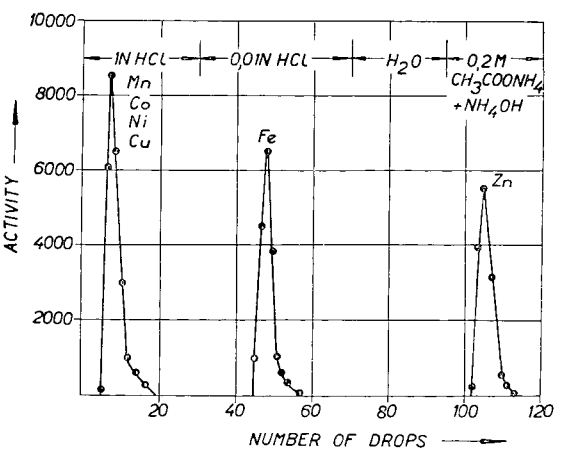

Fig. 1. Separation of (Mn,Co,Ni,Cu)–Fe–Zn.

of mixtures containing cobalt chloride and chlorides of other divalent metals from iron and zinc or cadmium. 0.03 ml samples of the solutions of chlorides in 1 *N* or 6 *N* HCl were used for the separation. Fig. 1 shows a chromatogram of the separation of certain chlorides from iron and zinc; manganese, cobalt, nickel and copper are eluted with 1 *N* HCl, iron with 0.01 *N* HCl, and zinc with 0.2 *M* ammonium acetate buffer (pH = 10), after washing the column with 0.75 ml of water.

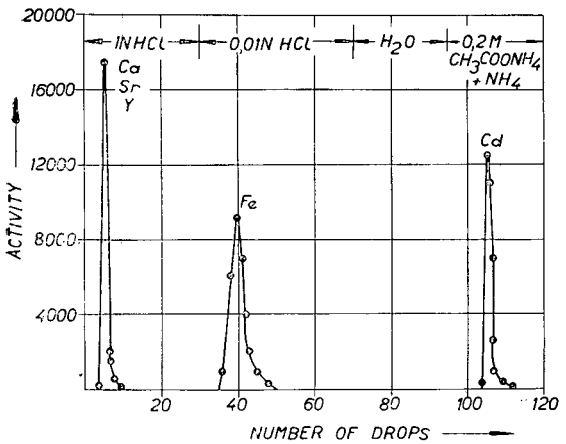

Fig. 2. Separation of (Ca,Sr,Y)–Fe–Cd.

Fig. 2. gives the chromatogram of separation of calcium and strontium chlorides and yttrium chloride from iron and cadmium; calcium, strontium and yttrium were washed with 1 *N* HCl, iron with 0.01 *N* HCl, and cadmium with 0.2 *M* ammonium acetate buffer (pH = 10), after washing the column with 0.75 ml of water.

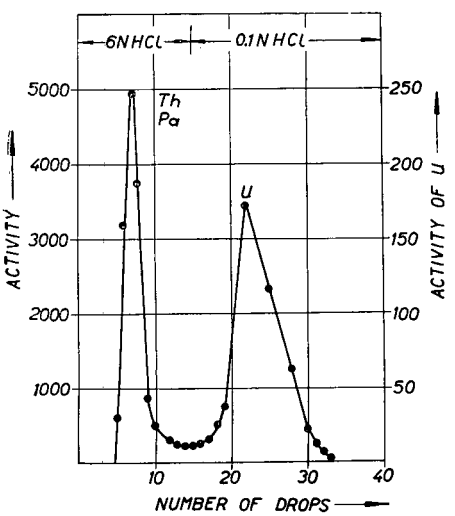

Fig. 3. Separation of (Th,Pa)–U.

In the case of thorium, protactinium, and uranyl chlorides it was shown that only uranyl chloride was adsorbed from 6 *N* hydrochloric acid, while thorium and protactinium do not adsorb and are not retarded by the column at all. Uranyl chloride could be eluted with 0.1 *N* HCl.

It was thus possible to obtain the carrier-free ^{234}Th (UX_1) isotope, formed as a result of an α-emission from the nucleus of ^{238}U. The UO_2Cl_2 preparation containing UX_1 was introduced into the column as a solution in 6 *N* HCl. Under these conditions the isotope of thorium was washed out in the first free volume of the column, but the uranium was adsorbed and could be eluted with 0.1 *N* HCl. Fig. 3 shows the chromatogram for the separation of thorium or protactinium from uranium.

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Book Reviews

Vitaminy, jejich Chemie a Biochemie (*Vitamine, ihre Chemie und Biochemie*), herausgegeben von JIŘÍ FRAGNER, 2 Bände, Nakladatelství Československé Akademie Věd, Prag, 1961, 1298 Seiten, Preis Kčs 149.00.

Der Herausgeber hat die Fortschritte der Vitaminforschung über eine Zeitspanne von etwa drei Jahrzehnten verfolgt und sich an der Ausnützung dieser Forschung in der Arznei- und Nahrungsmittelindustrie aktiv beteiligt. Die organisatorische Begabung und der Sinn für Zusammenarbeit, die er in dem industriellen Unternehmen und wissenschaftlicher Tätigkeit erwiesen hat, hat in diesem Werk zu einem äusserst ausgewogenen und durchdachten Ganzen geführt. Sechsundzwanzig Autoren (Chemiker, Biochemiker, Physiologen, Nahrungswissenschaftler, Technologen, Ärzte, Mikrobiologen und Analytiker) haben sich in der Auffassung, Unterteilung und Länge der Abschnitte dem Grundplan des Herausgebers untergeordnet; man spürt gar nicht, dass manchmal ein kurzer Abschnitt von etwa einem halben Dutzend Verfassern stammt. In jedem von den 20 Kapiteln des speziellen Teiles werden der Reihe nach die Geschichte, Nomenklatur, Chemie, Bestimmung, Vorkommen, Biochemie, Antivitamine und "gesellschaftliche Ausnützung" (in der Medizin, Nahrungsmittelindustrie und Landwirtschaft) beschrieben. Die Kapitel folgen einander alphabetisch nach den tschechischen Namen der betreffenden Vitamingruppe; der Herausgeber wollte dadurch ausdrücken, dass es keine allgemein anerkannte Klassifizierung der Vitamine gibt und dass jede rationelle Reihenfolge einseitig gewisse (oft nur hypothetische) Beziehungen unterstreichen möchte. Eine ausserordentliche Gewissenhaftigkeit kommt in den geschichtlichen Abschnitten in Erscheinung, es wird jedoch dem Historischen nicht zu Ungunsten des Modernsten Platz geschenkt. Die zehn Kapitel des allgemeinen Teiles besprechen die Frühgeschichte, Grundbegriffe, Nomenklatur, Beziehungen zwischen der chemischen Konstitution und biologischer Wirkung, Biogenese, komplexe biochemische Rollen (in Oxydoreduktionen, Aminosäureaktivierung, Transacylierung, Übertragen von C_1-Resten, biologischen Lichtreaktionen usw.), Bedeutung in der Nahrung, Vorkommen, Prinzipe der Bestimmung (einschliessend einer Verwertung der Chromatographie und Bioautographie) und gesellschaftliche Ausnützung der Vitamine. Ein anregendes Vorwort stammt von dem Internisten, Akademiker J. CHARVÁT.

Unter den Autoren der chromatographischen Abschnitte kommt am öftesten E. KNOBLOCH (Autor der Monographie über physikalisch-chemische Vitaminbestimmungsmethoden, 1956) und der Mikrobiologe A. ŠIMEK vor. Der Ausmass des Werkes erlaubte es nicht technische Einzelheiten anzugeben, jedoch die Grundzüge der Methoden und die wichtigsten Literaturquellen werden dargeboten. Die Gaschromatographie scheint ein wenig vernachlässigt zu sein. J. BLATTNÁ ist es gelungen, einen guten Überblick des wenig übersichtlichen Gebietes der Analyse von Provitaminen A auf bloss zwei Seiten zu schaffen. Umfangreiche Tabellen betreffend chromatographische Methoden bei der Bestimmung essentieller Fettsäuren wurden

CROMBIES Referat entnommen. Über die Papierchromatographie von Inosit wird ziemlich ausführlich referiert. Papierchromatographie der K-Vitamine wird unter dem Titel "Spezifität der Methoden" von J. BLATTNÁ besprochen, die chromatographische Methoden sind jedoch auch in diesem Kapittel, wie auch in einigen anderen, bei der Darstellung im chemischen Abschnitt erwähnt. Beim Vitamin D wird auf die Standardisierung der Adsorptionssäulen aufmerksam gemacht. Eine ganze Seite wird der Bioautographie der B_{12}-Vitamine gewidmet. Bei der Askorbinsäure wird auch die Bestimmung von Askorbigen besprochen. Es sei hier auch bemerkt, dass die papierchromatographische Methode von V. ŠANDA als Departage-Methode für Askorbinsäurebestimmung in die tschechoslowakische Staatsnorm übernommen wurde. Die Chromatographie der Blattsäure und ihrer Derivate wird in einem besonderen Abschnitt von K. SLAVÍK kritisch erwogen. Der Papierchromatographie der Thioktinsäure wird von J. ŠÍCHO eine Tabelle gewidmet. Chromatographische und elektrophoretische Methoden im Vitamin B_6-Gebiet überblickt E. KNOBLOCH; die Chromatographie von Riboflavin und Thiamin fasst er sehr knapp. Die Bedeutung der Chromatographie bei der Bestimmung von Tocopherolen geht auch aus der kurzen Übersicht hervor.

Die typographische Ausstattung ist im Einklang mit der Tradition des Verlages vortrefflich. Der graphische Künstler, J. LEDR, verdient erwähnt zu werden. Auch das Papier ist gut, was eher eine Ausnahme bei dem Verlag bildet. Von den störenden Druckfehlern möchte ich die Literaturangaben 106–111 auf S. 1076 nennen, wo man Autoren wie Singrkh oder u.091:rP.R finden kann. Das Nichtanführen von allen Autoren in einigen Zitationen ist ein Nachteil, die Angabe der Seite des Literaturverzeichnisses am Fuss jeder ungeraden Seite ein Vorteil. Die Tabelle von Askorbinsäurederivaten auf S. 713–720 ist unnötigerweise platzraubend gesetzt. Das Sachregister (41 Seiten) wurde von J. FRAGNER, D. REICHOVÁ UND A. ŽENÍŠEK sorgfältig verfasst. Der Preis ist fast unglaublich niedrig.

Der Rezensent freut sich auf das Neuerscheinen dieses Werkes in deutscher Sprache, das auf 1964 angekündigt wird. Man kann mit Recht erwarten, dass die sorgfältige Auswahl der neuesten Literatur, die zu den Merkmalen der hier besprochenen ersten Auflage gehört, fortgesetzt wird.

I. M. HAIS (Hradec Králové)

Stoichiometry and Structure, Part I, edited by M. J. SIENKO, W. A. Benjamin Inc., New York, 1964, 345 pp., price $ 3.25.

Stoichiometry and Structure is the first part of a two-volume work which is intended for use as a comprehensive introduction to the solution of chemical problems. It is concerned with general mathematical operations and their use in calculating atomic and molar properties, chemical composition and chemical reactions, problems which are generally met in the first part of a general chemistry course.

The title arises from the fact that the author introduces a number of simple

problems on the geometry of molecules, bond angles and bond lengths; in order to solve them almost one third of the book is devoted to a review of some mathematical notations including geometry and trigonometry. This is an unusual feature for a chemical calculations book. It is interesting, however, to find such emphasis given to structural chemistry in an elementary course.

The sequence of the topics is somewhat peculiar and very few would agree with the order selected by the author, *e.g.*, gases are treated almost at the end of the book, and galvanic cells and redox reactions are dealt with before explaining how to express the concentration of a solution and before mentioning chemical equilibrium, which is only discussed in the second volume.

The chapter devoted to solutions is quite unsatisfactory. Osmotic pressure is not even mentioned and the confusing term "apparent dissociation" for electrolytes is used in some examples.

Most problems are new, and anyone who teaches general chemistry is given the opportunity of finding a large number of good examples.

A. LIBERTI (Naples)

Practical Analytical Methods for Connective Tissue Proteins, by J. E. EASTOE AND A. COURTS, with a preface by Prof. A. G. WARD, E. & F. N. Spon Ltd., London, 1963, xiv + 145 pages, 11 figs., 11 tables, price cloth bound 42 s.

The authors combine the interests of such apparently disconnected fields as gelatin and glue technology and medical, nutritional and dental research, for they have worked in all of them. A biologist or physician who has come inadvertently in his line of research close to the glue and leather specialist will be puzzled by the diversity of analytical techniques and their modifications. The present book will help him to select well established and convenient methods for the preparation of connective tissue constituents (not only proteins, but mucopolysaccharides as well) and their physical and chemical characterization. Chromatographic methods are given their right place. Paper chromatography is not included, partly because it is too well known, partly because the authors consider it superseded by thin-layer chromatography. The MOORE AND STEIN ion-exchange chromatography and its modifications are described in considerable instrumental detail and the snags that may be encountered are given appropriate attention. Experimental procedures including Celite column chromatography are described for the estimation of the N-terminal groups of gelatin by the DNP method. A special chapter is devoted to thin-layer chromatography on Kieselgel G for the separation of amino acids and DNP-amino acids, and on Kieselguhr G for sugars. The invention of thin-layer chromatography is ascribed to STAHL (1956).

I. M. HAIS AND J. BARTOŠ (Hradec Králové)

Announcements

SECOND INTERNATIONAL SYMPOSIUM ON DRUGS AFFECTING LIPID METABOLISM

The European Society for Biochemical Pharmacology has organized, in collaboration with the University of Milan, the *Second International Symposium on Drugs Affecting Lipid Metabolism*, to be held in Milan on September 13th–15th, 1965.

The symposium will include plenary and short lectures by invited speakers and a limited number of original communications.

Requests for information, titles and original summaries of 200 words in English should be sent, before March 31st, 1965 to:

Prof. Rodolfo Paoletti, Scientific Secretary, Institute of Pharmacology, University of Milan, Via Andrea del Sarto 21, Milan. Telef. 719060.

J. Chromatog., 17 (1965) 204

SECOND INTERNATIONAL COURSE ON METHODS FOR LIPID RESEARCH

A NATO Advanced Study Institute on Methods for Lipid Research will be held at the University of Milan on September 16th–24th, 1965.

The course will consist of lectures and demonstrations held by European and American specialists. English will be the official language.

Participation is limited to 50 post-graduate applicants of any nationality, actively interested in lipid research, selected on a competitive basis. A good knowledge of English is essential. A limited number of fellowships for attendance at the course is available.

Applications for attendance and for the fellowships should be sent before March 31st, 1965, together with a *curriculum vitae* and a list of publications to:

Prof. Rodolfo Paoletti, Scientific Secretary, Institute of Pharmacology, University of Milan, Via Andrea del Sarto 21, Milan. Telef. 719060.

J. Chromatog., 17 (1965) 204

Errata

J. Chromatog., 15 (1964) 337

Table I, line 2 from bottom:

In "Commercial preparation (Lister)..." the word "Commercial" should be deleted.

J. Chromatog., 15 (1964) 379–392

Puromycin (Pur) does not belong to subclass Ic in Fig. 2 (p. 384) and in Table III (p. 386). It should be included in subclass IVb according to its R_F values in the principal solvent systems: 0.23 in 1, 0.29 in 2, zero in 3 and 4 (see Table II, Parts A and E, p. 383). The R_F values in the additional solvent systems I, J, K and L have not yet been determined because the author's stock of puromycin is exhausted.

J. Chromatog., 16 (1964) 311–326

Seite 313, Zeile 6:

Anstelle "...Gesamtdurchlässigkeit $D_{\lambda\varepsilon}$ von f bei einer..." muss es heissen "...Gesamtdurchlässigkeit $D_{\lambda\varepsilon}$ bei einer...".

Seite 320, Fig. 14:

Dem hellsten Fleck muss die Angabe 1.1 μg zugeordnet werden. Demnach wird die Konzentrationsangabe: 1.1 0.9 0.7 0.5 0.3 0.1 μg.

Bibliography Section

EDITORIAL

Although we desired to maintain the structure of the bibliography section along the established lines, we have felt it necessary to modify certain groups from time to time. Beginning with this issue, some changes will be made which do not involve the introduction or deletion of an entire group of compounds, but only concern the material treated within some groups. These changes will include the following features:

1. The main change concerns the lipids and their constituents, which have been dealt with so far in two groups. Section 11 (Organic Acids) included glycerides and simple lipids devoid of phosphorus in their molecule. Section 25 (Organic Phosphorus Compounds) featured more complex lipids containing phosphorus. In view of the experience that both types of compounds are more and more encountered in the presence of each other, all these compounds will in future be included solely in the Organic Acids Section. This Section will have two subdivisions:

11a. Organic acids.

11b. Lipids and their constituents.

2. Section 14 will contain saponins in addition to steroid glycosides.

3. Section 32 will be enlarged: in addition to Pharmaceutical Applications it will include forensic applications and papers dealing with the metabolism of drugs.

4. Section 34 will feature, in addition to Radioactive Compounds, also other isotopic compounds.

5. Paper and thin-layer chromatography have found a permanent use in the laboratory as routine methods. Both methods supplement each other conveniently and in many cases both of them are applied to the solution of a problem, also for a particular type of compounds, in order to increase the probability of identification. This situation, however, creates a problem in bibliographical documentation as to where the paper in question should be filed. Starting with this issue, we shall mark a paper featuring both methods at the end of the reference with "PC and TLC" and shall file it under that method which seems most important for the given task or where something new is introduced. A cross reference "See also... (TLC or PC)" will then be inserted at the corresponding group of compounds under the other method. A similar procedure will be introduced in the bibliography of gas chromatography if this is found to be promising.

6. Section 4 in the bibliography of gas chromatography will be further subdivided by adding 4f. Measurement of physico-chemical and related values.

K. MACEK AND J. JANÁK

Paper Chromatography

1. REVIEWS AND BOOKS

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NIIJIMA, K. AND OKUYAMA, T.: (Pigments in the Sakaguchi reaction. I. Comparison between the Sakaguchi reaction and the Voges–Proskauer reaction). *Seikagaku*, 35 (1963) 396–403; *C.A.*, 60 (1964) 6710b.

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8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

CHOPIN, J., BOUILLANT, M.-L. AND LEBRETON, P.: Isolement d'une nouvelle isoflavone naturelle: la méthyl-5 génistéine, extraite du bois de cytise (*Cytisus laburnum* L.). *Bull. Soc. Chim. France*, (1964) 1038–1042 — TL and paper chromatography.

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STRUCTURAL ANALYSIS OF ORGANIC SUBSTANCES BY MEANS OF HYDROGENATION COMBINED WITH GAS CHROMATOGRAPHY

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In recent years gas chromatography has been employed advantageously to the solution of problems involved in structural analysis of organic compounds. This development has been used as an adjunct to methods which in various ways modify the substance under test prior to its chromatographic treatment, so that from its behaviour before and after modification conclusions can be drawn on the arrangement of at least a part of the organic compound.

One such method is hydrogenation, which is usually carried out on a mixture of *e.g.* saturated and unsaturated compounds. The presence of unsaturated bonds is indicated by the disappearance of some chromatographic peaks[1–3]. In later work, hydrogenation of pure substances has been employed directly for identification purposes if they contain oxygen, nitrogen, or halogen[4–7], or for direct determination of the respective carbon skeleton[8–10]. In none of these procedures, however, has any attempt been made so far to compare the elution times of the hydrogenated and the non-hydrogenated product in such a way that from their relationship conclusions on the structural arrangement of the molecule of the substance under investigation may be drawn.

In the present work we have attempted to ascertain whether there was any relationship between the elution time and the number of double bonds in the molecule. In the chromatography of sterols, CLAYTON[11] has already found that a double bond causes a constant difference in the relative elution time. ACKMAN[12], who studied the relation between structure and elution volume of unsaturated fatty acids, found that the elution time changes depending on the position of the double bond; the larger the number of double bonds, the greater is this change.

It is generally known that saturated compounds exhibit shorter elution times than non-saturated compounds on polar stationary phases. This fact is frequently utilized, *e.g.*, in the separation of aromatic and aliphatic hydrocarbons, unsaturated and saturated acids, etc. If such separations are carried out and the relative elution time is calculated by dividing the elution time of the saturated substance by that of the unsaturated one, it was found that the relative elution time is proportional to the number of double bonds in the molecule.

For convenient and, at the same time, exact comparison of the elution times of these two types of substances in a single experiment, we have designed a simple hydrogenation apparatus which permits a simultaneous chromatographic record of both components.

EXPERIMENTAL

The principal part of the hydrogenation equipment is shown in Fig. 1 and consists of a layer of platinum catalyst C contained in tube A, which is heated in block B by means of the bodies I and J. The top end is fitted with a rubber closure E, through which the substance to be tested is fed by an injection syringe. Under this closure there is the hydrogen inlet CH. The hydrogen simultaneously serves as carrier gas. At the bottom end of the tube a needle, F, from an injection syringe is cemented so that it pierces into closure G of the chromatographic column H.

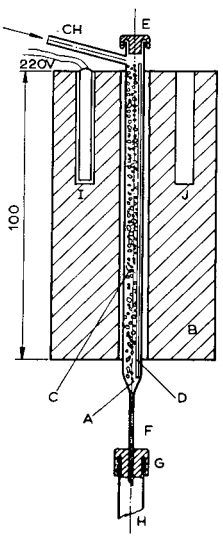

Fig. 1. Schematic drawing of the equipment for hydrogenation preceding chromatography, permitting simultaneous determination of the introduced non-hydrogenated and hydrogenated substances.

In order to obtain a simultaneous chromatograph of both the hydrogenated and the non-hydrogenated substance under the same conditions, one (or if required more) glass capillary of about 1 mm in diameter, extending from the top to the bottom end, is inserted into the catalyst layer. This arrangement permits about one half of the sample to pass through the catalyst and be hydrogenated, while the other half passes through the glass capillary without being affected.

Preparation of the catalyst

1 g of platinum is dissolved in aqua regia, evaporated to dryness, then HCl is added to remove HNO_3 and finally the evaporation to dryness is repeated. The residue is dissolved in about 15 ml of water and 10 g of pumice are poured into the solution contained in a porcelain dish. The mixture is dried to a brown coloration with constant agitation. The dried pumice is placed into a quarz tube, sealed with asbestos and reduced with hydrogen at a temperature of about 400°. The tube is emptied when cold.

Working conditions

It was found that hydrogenation proceeds best at a temperature of about 180°, and all the experiments described were performed at this temperature.

Gas chromatography was carried out in a CHROM I apparatus on a column of 85 cm in length, filled with Rysorb carrier with a stationary phase of 25 % of 3,5-dinitrobenzoyl ester of the butyl ether of triethylene glycol. Depending on the requirements, a column temperature of 68° and/or 150° was selected.

The flow rate of the carrier gas H_2 was 3.0 l/h. The relative elution time was calculated from the ratio of both elution times:

$$r_H = \frac{V_1 \text{ of non-hydrogenated substance}}{V_2 \text{ of hydrogenated substance}}.$$

DISCUSSION

From Tables I–VI it is obvious that the relationship between the number of –C=C– double bonds in the molecule and the logarithm of the relative elution time of the hydrogenated and non-hydrogenated substance is approximately linear

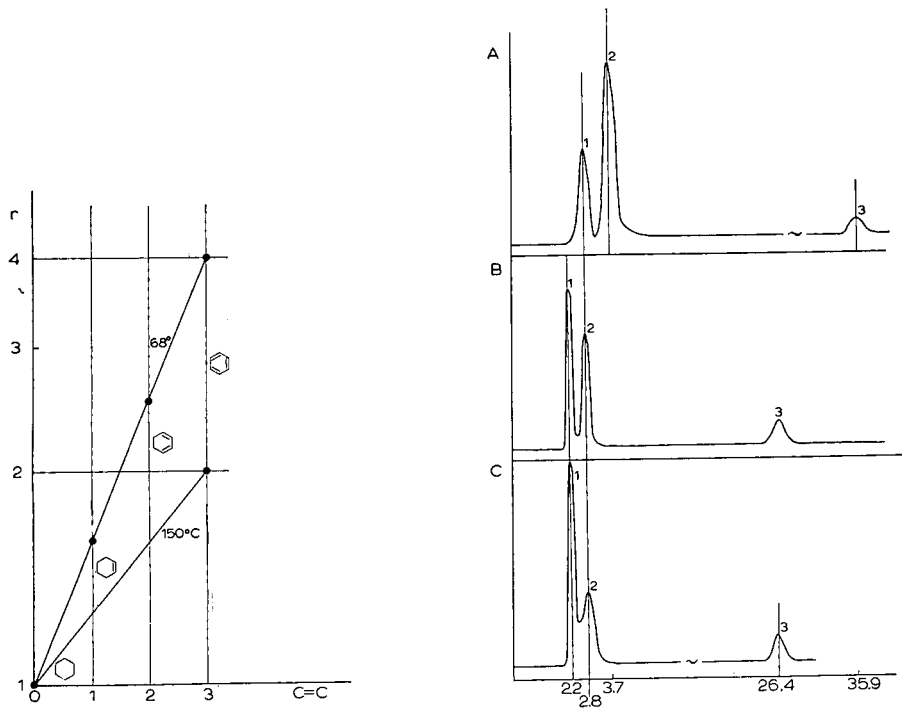

Fig. 2. Representation of the relationship between log r_H and the number of double bonds of benzene and its hydrogenation product at the various temperatures of the chromatographic column. Column filling as given in Table I.

Fig. 3. Hydrogenation of xylenes. A = *o*-xylene; B = *p*-xylene; C = *m*-xylene; (1) = *trans*-dimethylcyclohexane; (2) = *cis*-dimethylcyclohexane; (3) = xylene. Temperature of chromatographic column 68°. Filling 3,5-dinitrobenzoyl ester of butyl ether of triethyleneglycol.

TABLE I

RELATIVE ELUTION TIMES (r_H) OF MISCELLANEOUS HYDROCARBONS AND ESTERS AT VARIOUS TEMPERATURES ON A COLUMN WITH 3,5-DINITROBENZOYL ESTER OF BUTYL ETHER OF TRIETHYLENEGLYCOL

	$t = 68°$ r_H	$t = 150°$ r_H	*Number of double bonds reduced*
Cyclohexane	1.0	1.0	0
Cyclohexene	1.6	1.25	1
Methylcyclohexene	1.55		1
Dodecene		1.2	1
Cyclohexadiene	2.5		2
Thiophene	2.2		2
Benzene	4.0	2.0	3
Toluene	6.6	2.3	3
Ethylbenzene	4.8	2.3	3
o-Xylene	9.7	3.7	3
	12.4		
p-Xylene	9.4	3.6	3
	12.2		
m-Xylene	9.4	3.6	3
	12.2		
Cumene	3.4	2.4	3
p-Cymene	4.6	2.3	3
	5.2		
1,2,4-Trimethylbenzene	12.2		3
	15.0		
Butylbenzene	4.3	2.2	3
1,3,5-Trimethylbenzene		3.8	3
Methyl benzoate		2.6	3
Methyl phenylacetate		2.4	3
Styrene	12.0	4.1	4
α-Methylstyrene	8.8	4.3	4
p-Ethylvinylbenzene		3.8	4
Naphthalene		8.0	5
		10.5	
Methylnaphthalene		13.8	5
1,6-Dimethylnaphthalene		14.3	5
p-Divinylbenzene		6.3	5
1,6-Dimethyltetralin		2.9	3
		3.6	
Tetralin		3.1	3
		4.1	
Methyltetralin		2.9	3
		2.9	

(Fig. 2); however, the slope of this linear relationship depends on the temperature of the chromatographic column and the type of stationary phase. For this reason, two temperatures, 68° and 150°, were selected. The relatively greater scattering of the values obtained is usually due, on the other hand, to the occurrence of various isomers, whose elution time is influenced by the magnitude of the permanent dipole moment[13], and, on the other hand, to the formation of various isomers of the hydrogenated product (*cis*, *trans*). This circumstance, however, can be utilized for a more exact identification of the substance under test. For instance, *m*-xylene and *p*-xylene yield the respective *cis*- and *trans*-dimethylcyclohexanes, which, although they are

derived from the two different isomers, exhibit the same elution times. The ratio between the *cis* and the *trans* isomers, however, varies considerably (as is evident from Fig. 3), but is constant under the same working conditions, thus permitting the semi-quantitative estimation of the two components in the case of mixtures.

Despite all the effects mentioned, the variations in the relative elution times (r_H) are so small that the number of double bonds in various hydrocarbons can be reliably determined in the majority of cases (Fig. 4).

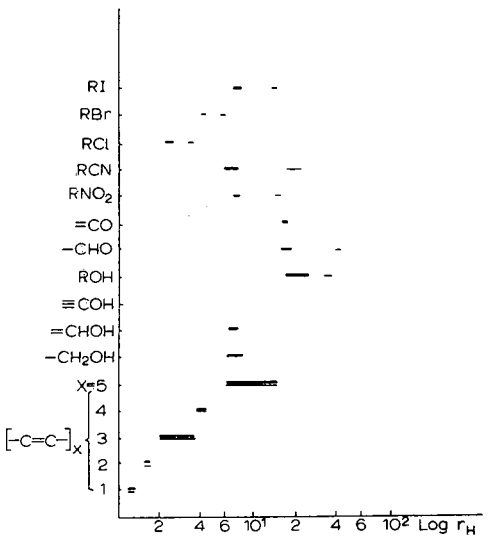

Fig. 4. Graphical representation of the magnitude of the relative elution time (r_H) for different numbers of double bonds and different functional groups that can be hydrogenated. Thin line: complete hydrogenation. Bold line: intermediate hydrogenation product corresponding to hydrogenation of the functional group.

The situation is sometimes complicated when the molecule contains functional groups, as well as double bonds, which can be hydrogenated, or groups which are split off under given conditions. For this reason, the analytical method described was applied to a series of compounds containing the groups $-CH_2OH$, $-OH$, $-CHO$, $=CO$, $-NO_2$, $-CN$, $-Cl$, $-I$ and $-Br$. The contribution of these groups to the relative elution time can also be investigated quantitatively since in the majority of cases it was possible to ascertain the intermediate products of the hydrogenation. The elution times are markedly increased by the presence of the above-mentioned functional groups. Tables II to IV present values showing how hydrogenation of the group or its splitting off influences the r_H. The diagram (Fig. 4) summarizes these results in such a way that according to the value of r_H conclusions can be drawn as to the number of double bonds as well as to the types of individual functional groups; thus, the substance under test can be identified in more detail. It is evident from the diagram that in certain cases the r_H's are equal for two different compounds. Further information can be obtained from the intermediate hydrogenation products present, or from the absolute elution time of the non-hydrogenated substance, which is usually longer for compounds with a larger molecule.

TABLE II

RELATIVE ELUTION TIMES (r_H) OF ALCOHOLS AND PHENOLS

	$t = 68°$ r_H	$t = 150°$ r_H	*Number of double bonds reduced*	*Group subjected to hydrogenation*	*Ratio corresponding to double bonds*
Methanol	15.2		0		
Ethanol	22		0		
Propanol	39		0		
n-Butanol	37		0		
Amyl alcohol	67		0	$-CH_2OH$	
Iso-amyl alcohol	38		0		
Hexyl alcohol	55	6.0	0		
Octyl alcohol	74	8.0	0		
3-Methylcyclohexanol		7.3 9.2	0		
4-Methylcyclohexanol		7.5 9.0	0	$=CHOH$	
Cyclohexanol	48	7.0	0		
tert.-Butanol	12.0	1.0	0	$\equiv COH$	
sec.-Butanol		6.1	0	$=CHOH$	
Phenol		16.6*	0		
o-Cresol		16.8*	0		
m-Cresol		22.1*	0	$-OH$	
p-Cresol		20.0*	0		
Phenol		33	3		2.0
o-Cresol		32	3		1.9
m-Cresol		35	3	$-OH$	1.6
p-Cresol		35	3		1.7
Furyl alcohol	7.4		2		
Allyl alcohol		1.1	1		
Furfural	2.2		2		

* Related to the elution time of benzene.

It must be pointed out, however, that hydrogenation of pyridine and its derivatives could not be achieved at the usual working temperature. The required effect was attained only after increasing the temperature up to 225°.

The presence of sulfur and halogens leads to a poisoning of the catalyst, usually requiring renewal of the reactor filling after a completed hydrogenation. Halogens are split off in this hydrogenation.

The hydrogenation of alcohols, which can be very easily differentiated, is also of interest. While primary and secondary alcohols have a comparatively high relative elution time, this time is essentially lower for tertiary alcohols. The comparatively great differences in the case of lower alcohols are caused by the fact that their hydrogenation results in highly volatile, paraffinic hydrocarbons, which exhibit short elution times, and the negligible differences in their elution times lead to considerable errors in their determination.

Ester groups are not hydrogenated and thus esters behave like hydrocarbons; however, they differ from hydrocarbons with the same number of double bonds by their essentially longer elution time. In the case of halogenated hydrocarbons, the

TABLE III

RELATIVE ELUTION TIMES (r_H) OF ALDEHYDES AND KETONES

	$t = 150°$ r_H	*Number of double bonds reduced*	*Group subjected to hydrogenation*	*Ratio corresponding to double bonds*
Propionaldehyde	18.4	0	–CHO	
Octylaldehyde	18.0	0		
Nonylaldehyde	18.6	0		
Decylaldehyde	26.4	0		
Benzaldehyde	16.1*	0	–CHO	
Cinnamaldehyde	16.0*	0		
o-Hydroxybenzaldehyde	16.6*	0		
p-Tolylaldehyde	17.0*	0		
Benzaldehyde	36.7	3	–CHO	2.3
Cinnamaldehyde	51.0	4		3.2
o-Hydroxybenzaldehyde	41.3	3		2.5
p-Tolylaldehyde	40.3	3		2.4
Cyclohexanone	17.0	0	=CO	
3-Methylcyclohexanone	16.3	0		
4-Methylcyclohexanone	17.5	0		
Methyl phenyl ketone	18.0	3	=CO	
Methyl phenyl ketone	8.0	0		
Cyclohexanone	2.2	0	=CO → –CHOH	
4-Methylcyclohexanone	2.0	0		
	2.4			
3-Methylcyclohexanone	1.9	0		
	2.4			

* Related to the respective aromatic hydrocarbon.

TABLE IV

RELATIVE ELUTION TIMES (r_H) OF NITRO COMPOUNDS AND NITRILES

	$t = 150°$ r_H	*Number of double bonds reduced*	*Group subjected to hydrogenation*	*Ratio corresponding to double bonds*
Nitrobenzene	7.5*	0	$-NO_2$	
Nitrotoluene	7.7*	0		
m-Tolunitrile	8.5*	0	–CN	
o-Tolunitrile	6.2*	0		
m-Tolunitrile	22.5	3	–CN	2.6
o-Tolunitrile	17.5	3		2.8

* Related to the elution time of aniline.

TABLE V

RELATIVE ELUTION TIME (r_H) OF HALOGENO-DERIVATIVES

	$t = 150°$ r_H	*Number of double bonds reduced*	*Group subjected to cleavage*
Chlorobenzene	3.4	3	–Cl
Chlorobenzene	2.3*	0	–Cl
Dichlorobenzene	6.5	3	2 –Cl
Bromobenzene	5.7	3	–Br
Bromobenzene	4.2*	0	–Br
Iodobenzene	13.2	3	–I
Iodobenzene	7.6*	0	–I
1,3,5-Trichlorobenzene	25.0	3	3 –Cl
1,2,4-Trichlorobenzene	25.0	3	3 –Cl

* Related to the elution time of benzene.

halogen present can be easily determined from the substantial differences in the relative elution times.

As already pointed out previously, the graph in Fig. 4 applies only to one temperature and to a certain stationary phase. If the experiments were carried out in parallel on different stationary phases, a further differentiation could be achieved, as is obvious from Table VI, which gives relative elution times on various stationary phases.

Another advantage of the method is the easy identification of intermediate hydrogenation products and the determination of the substance in question from the ratios of the elution times, and hence the mechanism of the reaction may be ascertained both qualitatively and quantitatively. This is clearly illustrated by Fig. 5.

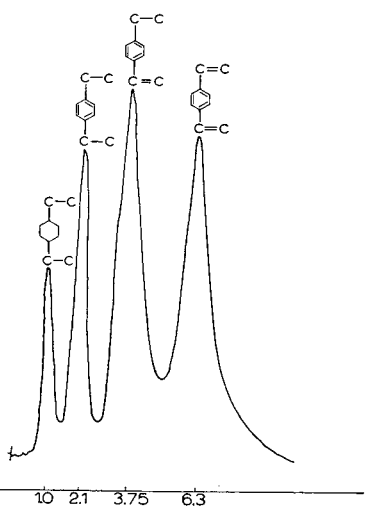

Fig. 5. Example of hydrogenation with identification of all intermediate products of hydrogenation of divinyl benzene.

TABLE VI

RELATIVE ELUTION TIME ON VARIOUS STATIONARY PHASES

$t = 150°$; Rysorb BLK.

	3,5-Dinitrobenzoyl ester 20% r_H	*Slovamin 7* 10% r_H	*Polywax 12.000* 20% r_H	*Asfalt EXP* 20% r_H
Benzene	2.0	2.2	1.9	0.0
Toluene	2.3	2.3	2.2	0.0
p-Xylene	3.6	2.6	2.7	0.0
Ethylbenzene	3.5	2.2	2.2	0.0
p-Cymene	2.3	2.3	2.3	0.0
Methyl benzoate	2.6	2.6	2.6	1.6
o-Tolunitrile	6.2	4.0	6.3	2.8
α-Methylstyrene	4.3	—	3.3	—
Cyclohexene	—	—	1.2	—
Cyclohexanol	7.0	7.8	13.0	7.2
p-Cresol	33.0	13.1	8.4	13.0
Phenol	33.0	14.6	10.2	19.1
3-Methylcyclohexanone	16.3	4.6	14.9	4.8
Mesitylene	3.8	2.6	—	—

SUMMARY

A method involving hydrogenation of a substance prior to chromatography has been developed and permits conclusions to be drawn on the presence of a certain number of double bonds, or certain functional groups simply from the ratio of the elution times of the hydrogenated and the non-hydrogenated substance. The elution times of both these substances are ascertained in a single experiment by means of a simple apparatus which is described in the paper.

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THE ANALYSIS OF OILS AND FATS BY GAS CHROMATOGRAPHY

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Gas–liquid chromatography has become a most important method for the determination of the fatty acid composition of natural oils and fats and the acidic part of complex lipids. The importance of the method has increased with the use of polyester stationary liquids, which enables a separation of the fatty acid methyl esters to be achieved on a basis of unsaturation[1,2].

In most of the published work on the determination of fatty acids, the acids were first converted to the corresponding methyl esters before separation by gas–liquid chromatography. Various investigators have studied the quantitative aspects of this conversion using methanol–hydrochloric acid[3], boron trifluoride–methanol[4], methylation of the silver salts of the acids by methyl iodide[5], and recently the results of a collaborative test have been published[6].

There have been many methods reported for obtaining the methyl esters of the fatty acids of oils and fats and these may be divided into two main groups, (a) transesterification of the glycerides in the presence of an excess of methanol, and (b) saponification of the glycerides with alkali, isolation of the free fatty acids and esterifying these acids. There are also many variations in procedure within these main groups. The usual catalyst for the transesterification is an alkali metal methoxide[2,7–11], but potassium hydroxide[12] and mineral acid[13] have also been used. Many variations, both in the reaction time and the strength of the alkali, in the saponification procedures have been reported[14–18].

It has been pointed out[18,19] that it is important to use mild conditions for the saponification of lipids since there is evidence that polyunsaturated acids may be partially isomerised during a saponification with concentrated alkali. Transesterification methods are therefore to be preferred. These latter methods are also less time consuming.

The purpose of the present investigation is to compare two different types of ionisation detectors with respect to their response to long-chain methyl esters and also to compare the following procedures for obtaining these methyl esters from oils and fats:

(a) Transesterification:

1. sodium methoxide catalyst, 40 min reaction time[10];
2. potassium hydroxide catalyst[12];
3. sodium methoxide catalyst, 4 h reaction time[8].

(b) Saponification-esterification:

4. saponification with 7.8 % ethanolic potassium hydroxide[19];
5. saponification with 0.5 *N* ethanolic potassium hydroxide[15];
6. cold saponification with 50 % aqueous potassium hydroxide[17].

EXPERIMENTAL

Preparation of methyl esters

(*a*) *Transesterification procedures*

1. A mixture of 5 g of the oil and 16 ml of 0.2 *N* sodium methoxide in methanol was heated under reflux for 40 min. The mixture was cooled, acidified with 2 *N* sulphuric acid and the resulting mixture was poured into an equal volume of cold, saturated sodium chloride solution. The methyl esters were extracted with 3 × 10 ml portions of light petrol (b.p. 40–60°) and the combined extracts were washed with small portions of ice-cold water until the washings were neutral. The light petrol extract was dried for 30 min over anhydrous sodium sulphate and, after filtration, the bulk of the light petrol was removed by distillation.

2. A mixture of 5 g of the oil and 25 ml of 0.2% potassium hydroxide in methanol was heated under reflux until the mixture was homogeneous, and then for a further 10 min. The mixture was cooled, poured into an equal volume of cold water and the methyl esters extracted with 3 × 20 ml portions of diethyl ether. The combined extracts were washed with small portions of water until the washings were neutral. The ether extract was dried over anhydrous sodium sulphate and, after filtration, the bulk of the ether was removed by distillation.

3. A mixture of 2 g of the oil and 35 ml of methanol was heated under reflux for a few minutes, 3.5 ml of 1% sodium methoxide in methanol were added and the resulting solution heated under reflux for 4 h. The mixture was cooled, poured into an equal volume of water and the methyl esters obtained by extraction into ether as in procedure 2 above.

(*b*) *Saponification–esterification procedures*

4. A mixture of 10 g of the oil and 50 ml of 7.8% potassium hydroxide in 95% ethanol was heated under reflux for 3 h. The resulting solution was poured into an equal volume of cold water and the unsaponifiable material removed by extraction with ether. The aqueous layer, containing the potassium salts of the fatty acids was acidified with 2 *N* sulphuric acid and the free fatty acids were extracted with 3 × 10 ml portions of diethyl ether. The combined ether extracts were dried for 30 min over anhydrous sodium sulphate, and, after filtration, the ether was removed by distillation.

The fatty acids obtained were esterified by heating under reflux for 4 h with 50 ml methanol containing 1% by volume sulphuric acid. The resulting solution was poured into an equal volume of water and the methyl esters were extracted with 3 × 10 ml portions of diethyl ether. The combined ether extracts were washed with an aqueous sodium bicarbonate solution and finally with water. The ether extract was dried for 30 min over anhydrous sodium sulphate and, after filtration, the bulk of the ether was removed by distillation.

5. A mixture of 5 g of the oil and 40 ml of 0.5 *N* potassium hydroxide in 95% ethanol was heated under reflux for 2 h. The resulting solution was poured into an equal volume of cold water. The fatty acids were obtained and converted into their methyl esters by the same method as in procedure 4.

6. 10 g of the oil was added, with vigorous stirring, to a solution of 2.6 g potassium hydroxide in 2.5 ml of water. The reaction was exothermic, and, after cooling the reaction vessel, 0.1 ml of ethanol was added. The resulting mixture was allowed

to stand overnight at room temperature. The solid mass obtained was dissolved in 150 ml of cold water and this solution was processed to give the free fatty acids and then their methyl esters by the method used in procedure 4.

Gas–liquid chromatography

The methyl esters of the fatty acids were separated on two different gas chromatographs, a Perkin Elmer 800 (PE 800) instrument with a dual flame ionisation detector, and a Pye Argon Chromatograph (PAC) with a strontium-90 ionisation detector. The operating parameters were:

PE 800. 6 ft. × $^1/_8$ in. O.D. stainless steel column packed with butanediol succinate (BDS) on HMDS Chromosorb W (80–100 mesh) (8:92, w/w); for the linseed and corn oil esters the column temperature was programmed from 180° to 210° at a rate of 3.3 degs/min; for the coconut oil esters the column temperature was programmed from 150° to 210° at a rate of 6.7 degs/min. The flash heater temperature was 300°; flow-rate of nitrogen was 30 ml/min. Samples were injected onto the chromatographic column by means of a Hamilton micro-syringe.

PAC. 4 ft. × $^1/_4$ in. O.D. glass column packed with PEGA on acid-washed Celite 545 (100–120 mesh) (10:90, w/w); for the linseed and corn oil esters the column temperature was 180°. It was not possible to estimate all the methyl esters from coconut oil at one column temperature; the lower molecular weight esters were separated at 140° and the higher molecular weight esters were separated at 180° and a quantitative relationship between the chromatograms obtained at the two temperatures was established using the area of the methyl palmitate peak[3,20]. Argon was the carrier gas used at a pressure of 10 lb./sq.in., the applied voltage was 1250 V. Samples were placed on the chromatographic column with a micro-dipper pipette.

The areas under the peaks were obtained by multiplying the peak height by the width at half peak height[21], and the percentage areas obtained by the internal normalisation technique.

RESULT AND DISCUSSION

It has been shown[22] that, for accurate quantitative results using gas chromatographic methods, calibration of the detector with pure compounds is essential. ETTRE AND KABOT[23] have recently published results of the response of the PE 800 dual flame ionisation detector to fatty acid methyl esters. These workers used BDS-coated open tubular columns and found that the areas per cent were proportional to the weight per cent of the esters. In the present work this has been confirmed when conventional packed columns were used (Table I).

The response of a β-ray ionisation detector appears to depend on the geometry of the cell and the operating conditions employed. The variations in response to fatty acid methyl esters have been discussed by ACKMANN AND BURGHER[24]. The response factors obtained in the present work are shown in Table II, and it was found that the response factors for the lower molecular weight fatty esters are dependent on the applied voltage. It is therefore necessary to calibrate the detector for each applied voltage setting.

Pure samples of linoleic and linolenic methyl esters were not available to the present authors for calibration purpose but since the relative amounts of these two

TABLE I

RESPONSE OF A FLAME IONISATION DETECTOR TO FATTY ACID METHYL ESTERS

Ester	*Weight response relative to methyl palmitate* (= 100)	
	a	*b*
Methyl octoate	99	97
Methyl decoate	100	101
Methyl laurate	100	100
Methyl myristate	99	98
Methyl palmitate	100	100
Methyl stearate	101	101

a = present work.
b = results of ETTRE AND KABOT[23].

esters are similar on the two different instruments, it may be concluded that the responses of the two different ionisation detectors to these esters were similar.

The results obtained for the relative amounts of the major constituent fatty acids of linseed oil, corn oil and coconut oil are shown in Table III. It can be seen that, for linseed oil and corn oil, good agreement is obtained for all six procedures used for obtaining the methyl esters. Also, there is good agreement of the results obtained on the two different instruments. For the coconut oil there is good agreement of the results obtained on the two different instruments. There is also good agreement of the results obtained by five of the procedures for obtaining the methyl esters but the relative proportion of lauric acid is much lower in the methyl esters obtained by transesterification procedure 3.

In this last mentioned procedure it was found that, when the reaction mixture was poured into water and the methyl esters extracted by ether, the aqueous layer remained cloudy and it was thought probable that some material was not being extracted by the ether. The aqueous layer was acidified, solid sodium chloride was added until the solution was almost saturated, and the resulting solution was ex-

TABLE II

RESPONSE OF A β-RAY IONISATION DETECTOR TO FATTY ACID METHYL ESTERS

Ester	*Weight response relative to methyl palmitate* (= 100) *in dependence on applied voltage* (*V*)			
	1000	*1250*	*1500*	*1750*
Methyl octoate	60	85	98	101
Methyl decoate	66	90	100	101
Methyl laurate	72	93	102	101
Methyl myristate	89	98	99	100
Methyl palmitate	100	100	100	100
Methyl stearate	100	101	103	105

tracted with ether. After this extraction the aqueous layer was clear. This ether solution was dried and chromatographed on the PE 800 instrument. It was found that this solution contained a greater proportion of lauric acid than the original solution of methyl esters obtained by procedure 3.

TABLE III

RELATIVE AMOUNTS OF THE MAJOR CONSTITUENT FATTY ACIDS

Acid	*Flame ionisation detector*						*β-Ray ionisation detector*					
	Transesterification			*Saponification-esterification*			*Transesterification*			*Saponification-esterification*		
	1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*	*4*	*5*	*6*
Linseed oil												
16:0	5.6	5.8	6.2	6.3	5.8	5.8	5.3	5.4	5.4	5.7	5.9	5.8
18:0	3.7	3.6	3.5	3.5	3.6	3.7	3.9	3.8	4.3	3.5	3.8	3.7
18:1	18.6	18.8	18.7	18.3	18.1	18.1	18.4	18.2	18.7	18.4	18.1	18.2
18:2	13.2	13.2	13.5	13.8	13.8	13.7	13.3	13.4	13.7	13.4	13.4	13.7
18:3	58.9	58.6	58.1	58.1	58.7	58.7	59.1	59.2	57.9	58.9	58.8	58.6
Corn oil												
16:0	11.4	11.2	11.7	12.1	11.7	11.5	11.8	11.7	11.6	12.0	12.0	11.5
18:0	2.0	1.8	2.2	2.0	1.9	2.0	2.2	2.0	1.9	2.0	2.1	2.0
18:1	32.0	32.1	32.6	31.7	31.5	32.2	31.8	32.2	32.8	32.1	31.6	32.0
18:2	54.6	54.9	53.5	54.2	54.9	54.3	54.2	54.1	53.7	53.9	54.3	54.3
Coconut oil												
6:0	0.6	0.6	1.1	0.5	0.6	0.6	(0.6)*	(0.6)*	(1.1)*	(0.5)*	(0.6)*	(0.6)*
8:0	8.0	9.6	11.3	9.5	9.2	9.0	8.9	10.9	11.5	9.1	10.4	10.9
10:0	5.8	6.3	5.8	6.8	6.8	6.9	5.5	5.9	5.5	5.5	5.7	5.8
12:0	50.5	50.0	45.7	50.6	49.9	48.7	51.6	49.8	44.8	51.6	50.3	49.5
14:0	19.1	17.8	19.4	18.2	18.4	18.5	19.0	18.5	19.1	18.8	18.6	18.5
16:0	7.2	7.0	8.2	6.9	7.1	7.5	7.3	6.7	8.3	7.0	6.9	7.2
18:0	2.2	2.1	2.3	1.8	1.9	2.3	2.1	2.0	2.5	2.2	2.0	2.0
18:1	5.3	5.1	5.3	4.5	5.0	5.2	4.4	4.5	5.7	4.6	4.6	4.7
18:2	1.3	1.4	1.3	1.2	1.1	1.3	0.6	1.1	1.5	0.7	0.9	0.8

* Results obtained on flame ionisation detector.

These two solutions of methyl esters were combined and it was found that the relative proportions of the methyl esters in this combined solution were similar to those found by the other procedures. A sample of the coconut oil was then transesterified by procedure 3, modified to include the acidification of the reaction mixture and saturation with sodium chloride before ether extraction. The resulting solution of the methyl esters was chromatographed. The results of these experiments are summarised in Table IV. KAUFMANN AND MANKEL[13] have also reported loss of lower molecular weight fatty acids during the extraction stage and they have overcome this loss by extraction with a mixture of docosane and light petrol.

The amounts of some of the minor constituent fatty acids of linseed oil and corn oil obtained on the PE 800 instrument are shown in Table V. The amounts of lauric and myristic acid obtained by all six procedures are similar but there are large

TABLE IV

RELATIVE AMOUNTS OF FATTY ACIDS OBTAINED FROM COCONUT OIL

Acid	*a*	*b*	*c*	*d*
6:0	1.0	0.6	0.8	0.9
8:0	12.8	4.9	8.7	10.4
10:0	4.8	7.6	6.3	6.6
12:0	43.5	56.4	50.1	49.6
14:0	19.4	17.4	18.4	18.0
16:0	7.8	6.3	7.0	7.0
18:0	3.0	1.6	2.3	2.0
18:1	6.4	4.1	5.2	4.5
18:2	1.3	1.1	1.2	1.0

a = obtained by procedure 3.
b = extracts of the aqueous layer of procedure 3.
c = extracts a and b combined.
d = obtained by modified procedure 3.

variations in the amounts of the other acids, *e.g.* the amount of the triunsaturated C_{14} acid in corn oil varies from 0.03 % (procedure 1) to 0.95 % (procedure 4). The total amounts of the minor constituent fatty acids of corn oil found by the transesterification procedures are much lower than the amounts obtained by the other procedures.

When the coconut oil methyl esters were chromatographed on the PEGA column a peak was obtained which had a retention time of 0.86 relative to methyl stearate (= 1.00) and an equivalent chain length of 17.50. This peak is probably due

TABLE V

PERCENTAGE AMOUNTS OF THE MINOR FATTY ACID CONSTITUENTS

Acid	*Procedure*					
	1	*2*	*3*	*4*	*5*	*6*
Linseed oil						
12:0	0.08	0.08	0.07	0.08	0.08	0.07
12:unsat.	0.01	0.02	0.01	0.01	0.02	0.04
12:unsat.	0.02	0.04	> 0.01	0.03	0.04	0.05
13:0	0.02	0.04	> 0.01	0.04	0.02	> 0.01
14:0	0.04	0.04	0.05	0.04	0.04	0.02
14:3	0.17	0.11	0.03	0.02	0.04	0.03
Total	0.34	0.33	0.18	0.22	0.24	0.21
Corn oil						
10:0	0.03	0.03	0.02	0.09	0.02	0.02
11:0	0.01	0.03	0.03	0.09	0.09	0.09
12:0	0.33	0.30	0.29	0.37	0.33	0.35
12:unsat.	0.01	0.21	0.15	0.32	0.30	0.35
13:0	0.06	0.43	0.10	0.83	0.81	0.99
14:0	0.12	0.09	0.10	0.09	0.10	0.13
14:3	0.03	0.17	0.10	0.95	0.56	0.77
Total	0.59	1.26	0.79	2.74	2.21	2.70

to the presence of a triunsaturated C_{16} methyl ester. The amounts of this component in the methyl esters obtained by the different procedures varied considerably:

Procedure	1	2	3	4	5	6
Percentage 16:3	0.71	0.01	0.01	0.36	0.07	0.09

When the coconut oil methyl esters obtained by procedures 4 and 5 were chromatographed on the BDS column peaks were obtained for the monounsaturated C_8, C_{10}, C_{12}, C_{14} and C_{16} methyl esters and the amounts of these esters are shown in Table VI.

TABLE VI

PERCENTAGE AMOUNTS OF MONOUNSATURATED ACIDS IN COCONUT OIL

Acid	*Procedure*	
	4	*5*
8:1	0.5	> 0.1
10:1	0.3	0.1
12:1	2.5	1.3
14:1	1.0	0.5
16:1	0.3	0.2

These peaks were barely discernible in the chromatograms of the methyl esters obtained by the other procedures. The amounts of monounsaturated C_{16} fatty acid found by KAUFMANN AND MANKEL[13] in samples of sesame oil and maize oil vary by a factor of 2 depending on the procedure used to obtain the methyl esters from these oils.

CONCLUSIONS

The results given here show that gas chromatography is a convenient method for determining the fatty acid content of oils and fats. When mixtures of fatty acids contain acids with a range of molecular weights then a gas chromatograph with temperature programming simplifies the operations involved. The six procedures for obtaining fatty acid methyl esters from oils and fats give similar results for the fatty acids present in major amounts so long as precautions are taken to obviate the loss of lower molecular weight fatty acids. Further work is obviously necessary to account for the varying results obtained by the different procedures for the minor constituent fatty acids.

SUMMARY

A comparison is made of six procedures of obtaining methyl esters of fatty acids from oils and fats and also of two gas chromatographic instruments for the separation of these esters. The responses of a dual flame ionisation and a β-ray ionisation detector to long-chain fatty acid methyl esters are discussed.

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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS

A METHOD FOR THE DETERMINATION OF C_1 TO C_6 ACIDS IN BIOLOGICAL MATERIAL

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INTRODUCTION

The classical work of JAMES AND MARTIN[1] demonstrated that the separation and quantitative estimation of saturated monocarboxylic acids, up to and including dodecanoic acid, could be achieved by means of gas–liquid chromatography (GLC). However, routine determination of volatile acids present in biological materials has remained a formidable task. Such acids are usually separated from materials in which they occur by steam distillation and recovered as an aqueous solution of their sodium salts. Part of the difficulty entailed in the analysis of these acids by GLC lies in the requirement, with most column packings, that they be applied in free form and in an anhydrous state. In the procedure of JAMES AND MARTIN[1] a micro-partition column was employed to convert sodium salts to an anhydrous ethereal solution of the free acids, which was then distilled onto the chromatographic column for analysis. It was claimed that the method gave quantitative results. Often, however, difficulties have been experienced with the method and various alternative procedures have been proposed. See, for example, MCINNES[2], GEHRKE AND LAMKIN[3], and SHELLEY, SALWIN AND HORWITZ[4]. SMITH[5] demonstrated the possibility of analysing aqueous solutions of free fatty acids by GLC, using a stationary phase of Tween 80 on Celite but no means of applying the process to sodium salts was described.

In the present communication, a simplified procedure for the analysis of mixed sodium salts of volatile fatty acids by means of GLC is described. It gives quantitative results, with complete separation of saturated monocarboxylic acids from formic to caproic, inclusive. The method is based upon the observation that a liquid phase consisting of behenic and orthophosphoric acids tolerates the introduction of a small amount of water with the sample of acids. JACKSON[6] showed that this liquid phase gave excellent separation of volatile acids from C_1–C_5, at 135°, with helium as the carrier gas, but found some evidence of partial decomposition of formic acid under the conditions used. We have since established that such decomposition is prevented by using wet nitrogen as the carrier gas.

A gaseous phase containing water vapour precludes the use of a thermal conductivity detector. Flame ionisation detectors, which may be used with a wet gas, unfortunately do not respond to formic acid. The response of an argon ionisation

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detector to organic compounds is damped by the presence of water vapour in the carrier gas and, although this type of detector has not been tried for our purpose, it therefore appears unlikely that it could be used satisfactorily. Thus, in the method of analysis described below, titration was chosen as the means of detecting acids eluted from the column. As an alternative to the photo-electric titration apparatus described by JAMES AND MARTIN[1] and MCINNES[2], the present authors have found that a glass electrode, pH-stat assembly is suitable as a recording detector for the acids.

EXPERIMENTAL

Apparatus

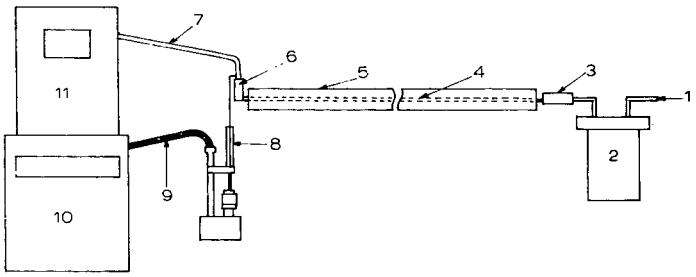
The chromatographic apparatus used for this study was constructed in the laboratory (see Fig. 1). It consisted of a water bubbler through which the carrier gas (nitrogen) could be passed before entering the column, a detachable sample heater, a straight chromatographic column, 0.4 cm I.D. by 125 cm long, within a cylindrical heating oven, a titration vessel and the automatic titrator. With the exception of the oven, borosilicate glass was used for all parts. Demountable joints were secured by clamps or spring-loading so as to withstand an internal pressure of 25 lb./sq. in. The heating element for the sample heater consisted of an externally wound nichrome ribbon (40 Ω total resistance) which was covered by an insulating layer of fibreglass adhesive tape. Operating at an applied potential of 18–20 V gave an internal temperature between 140° and 160°. The oven walls consisted of two aluminium tubes approximately 125 cm long, an inner tube (2.5 cm diameter) being located coaxially with the outer (6.4 cm diameter) by means of cork spacing rings. The inner tube was wound with a 150 W heating tape, 183 cm long by 5 cm wide, and the space between the heating tape and the outer aluminium tube was packed with asbestos. The partitioning column was supported in the oven also by means of cork spacers, the one at the input end being split in order to facilitate assembly and dismantling. Within the oven, the column was enclosed in a loosely fitting glass tube which enabled thermocouples to be held in a suitable position for column temperatures to be determined. Analyses were carried out with (rising) temperature programming. Adequate temperature control was obtained by means of a variable transformer. Although this method of control did not give linear programming, the temperature–time curve was sufficiently reproducible. Any effects of variations in this were insignificant.

Emerging acids were determined by means of the recording pH-stat assembly (Radiometer, Copenhagen: Titrator TTT1c, Titrigraph SBR2c and Syringe Burette SBU1c). When 0.02 *N* alkali was used as titrant, the limit of readability on a titration curve corresponded to $\pm$ 0.01 μmole.

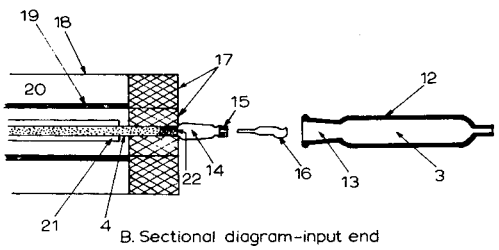
Since the gas stream from the column tends to heat the titration cell contents, an upward slope in the baseline may be evident. The extent of the rise in baseline may be limited by starting an analysis with the titration liquid at 35° and making use of the cooling effect of the nitrogen stream through the auxiliary bubbler which is employed to mix the cell contents. Allowance may readily be made for a rising baseline when reading results from the curve.

Stationary phase

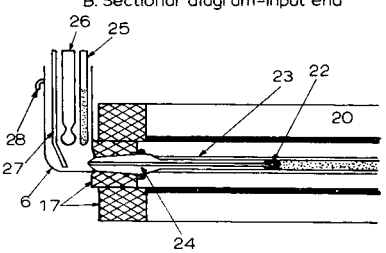
The stationary phase consisted of behenic acid, 20 g, orthophosphoric acid, 4 g,



A. Chromatograph assembly (not to scale)



B. Sectional diagram-input end



C. Sectional diagram-titration end

Fig. 1. Gas–liquid chromatographic apparatus (Figs. 1B and 1C are approx. $^1/_4$ size):

1. Gas inlet.
2. Water bubbler.
3. Sample heater.
4. Chromatographic column.
5. Column oven.
6. Titration vessel.
7. Electrode leads.
8. Micro-burette.
9. Flexible drive.
10. Recorder.
11. pH meter-titrator.
12. Insulated heater (40 Ω).
13. Ground glass socket-B10.
14. Modified glass cone-B10.
15. Silicone rubber plug.
16. Glass sample boat.
17. Cork spacers.
18. Aluminium tube.
19. Aluminium tube with heating tape (150 W).
20. Asbestos packing.
21. Glass tube for thermocouples.
22. Glass yarn plug.
23. Capillary end of column.
24. Glass cone and socket-B7.
25. Calomel electrode.
26. Glass electrode.
27. Bubbling tube.
28. Vacuum connection.

on acid-washed Chromosorb W (F & M Scientific Corporation) 80–100 mesh, 100 g. This mixture gives excellent separation of C_1–C_6 monocarboxylic acids, with wet nitrogen as the carrier gas. It has, moreover, a substantial tolerance of water vapour in the gaseous phase, a feature of basic importance to the technique to be described here. Reagent grade (syrupy) phosphoric acid was used as received but laboratory grade behenic acid was re-crystallized from acetone. The column packing was prepared by dissolving the acids in acetone, adding the Chromosorb, then evaporating the solvent by heating over a steam bath with constant stirring. Columns were packed, with the aid of a vibrator, to a density such that, at 90–100°, a flow rate of 50 ml/min

was obtained with a head pressure of 18–20 lb./sq. in. Before use, the packed column was conditioned by heating overnight at 100° while passing through it a slow stream (10 ml/min) of wet nitrogen. Omission of this step resulted in low recoveries of formic acid, and high retention volumes with incomplete separation of formic and acetic acids during the first few analyses. The reason for this beneficial effect of hydration of the column packing on its performance is obscure.

Sample preparation

Volatile acids were distilled from biological material according to FRIEDEMANN[7] and titrated to the phenolphthalein end point with NaOH (0.1 *M*). One ml excess of the alkali was then added and the sodium salts solution concentrated by boiling. It was then made up to a volume such that the total concentration of acids was approximately *M*. A sample of this solution (10 μl) was transferred to a glass boat (see Fig. 1) by means of a micro-syringe. The syringe needle had a square cut tip and the last few mm of its outer surface were smeared lightly with silicone grease. Gentle suction was applied to the boat while it was warmed carefully with a micro-flame to dry the sample. A further 10 μl volume of the salts solution was then placed in the boat and similarly dried, the process being repeated until a sufficient quantity of salts had accumulated (5–10 μmoles). Best results were obtained when the salts were dried in a compact mass. Drying to the extent of removing water of crystallization from the salts was found undesirable since this usually led to loss by spattering. Boats containing dried salts could be stored, in a desiccator, without loss of fatty acids.

Chromatography

The column temperature was raised to 90° and maintained at this level by appropriate adjustment of the applied voltage. At the same time, the sample heater was switched on (20 V). Reduced pressure equal to 0.5 atm was applied to the column via the titration vessel and a boat containing a sample of dried salts was attached to the input end by means of the perforated silicone rubber plug. Phosphoric acid solution (15 μl of 30 % w/v) was dropped onto the salts in such a manner that the whole sample was wetted at the same time. The sample heater was then attached to the column and suction continued for 5 min. At the end of this period the vacuum was gradually replaced by a flow of wet nitrogen at 8–10 ml/min. Five minutes later, this flow rate was increased to 30 ml/min and the oven heater voltage raised to 180 V. Acids were neutralized as they emerged from the column and the volume of alkali (0.02 *M*) consumed was recorded with the pH-stat assembly. The volume of liquid in the titration vessel was 15 ml and the end-point setting pH 7.5.

Formic acid, if present, emerged from the column about 5 min after increasing the nitrogen flow to 30 ml/min. The order of emergence of the acids was the same as found by JAMES AND MARTIN[1] using stearic acid/silicone columns. When *n*-butyric acid appeared (31–32 min) the nitrogen flow was increased to 40–50 ml/min in order to keep the time required for the analysis to a minimum (60–70 min for caproic acid). The various acids present in the column could be identified by their time of emergence under standard conditions of operation and the amounts read directly in micro-equivalents from the chromatogram by means of the chart graduation lines (see Fig. 2).

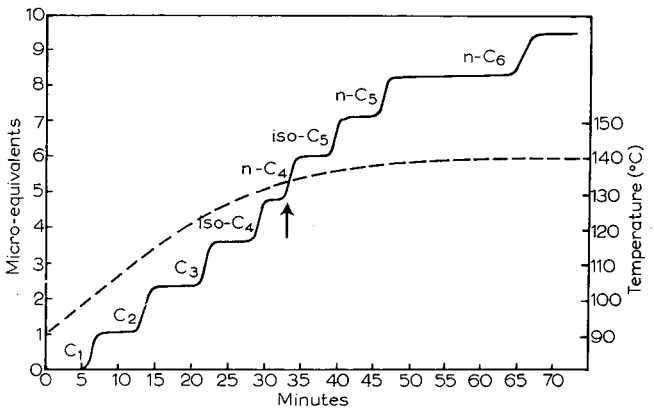


Fig. 2. Gas–liquid chromatogram from a synthetic mixture of sodium salts of volatile fatty acids, using a 125 cm behenic acid–phosphoric acid column with wet nitrogen as carrier gas. Acids: C_1 = formic; C_2 = acetic; C_3 = propionic; iso-C_4 = isobutyric; n-C_4 = n-butyric; iso-C_5 = isovaleric; n-C_5 = n-valeric; n-C_6 = n-caproic. ——— alkali consumed; – – – column temperature. For other details see Fig. 1 and text.

RESULTS

Evaluation of the method

Fig. 2 illustrates the chromatogram obtained from a synthetic mixture of sodium salts of eight acids within the range C_1–C_6. It can be seen that the titration curves display an almost complete lack of tailing. The isovaleric curve does lack symmetry, probably because of contamination of the sample used with α-methylbutyric acid (see JAMES AND MARTIN[1]). These two isomers are not completely separated on a 125 cm behenic acid–phosphoric acid column under the conditions employed.

The accuracy and reproducibility of results obtainable with the method were examined by replicate analyses of 10 μl portions of a synthetic mixture of sodium

TABLE I

ANALYSIS OF A SYNTHETIC MIXTURE OF VOLATILE FATTY ACIDS (6 REPLICATES) BY GAS–LIQUID CHROMATOGRAPHY ON A BEHENIC ACID–ORTHOPHOSPHORIC ACID COLUMN

Acid determined	*Found (mean μmoles)*	*Standard deviation**
Formic	0.827	±0.008
Acetic	0.915	±0.011
Propionic	0.861	±0.012
Isobutyric	0.843	±0.009
n-Butyric	0.861	±0.010
Isovaleric	0.803	±0.005
n-Valeric	0.788	±0.008
n-Caproic	1.035	±0.006
Total acids	6.935	±0.025
Total acids taken (μmoles)	7.020	
Total recovery (%)	98.9	±0.35

* Differences between the standard deviations for individual acids were not significant.

salts, the total acid content of this solution being determined by steam distillation and titration of a larger sample. Results from this investigation shown in Table I indicate that the method possesses high levels of accuracy and precision. No evidence of specific differences in recoveries of individual acids is apparent.

Application of the method

The method described has been used by the authors as a means of estimating components of volatile acidic fractions from silages and rumen contents. Fig. 3 illustrates the chromatogram obtained with material from a poorly preserved,

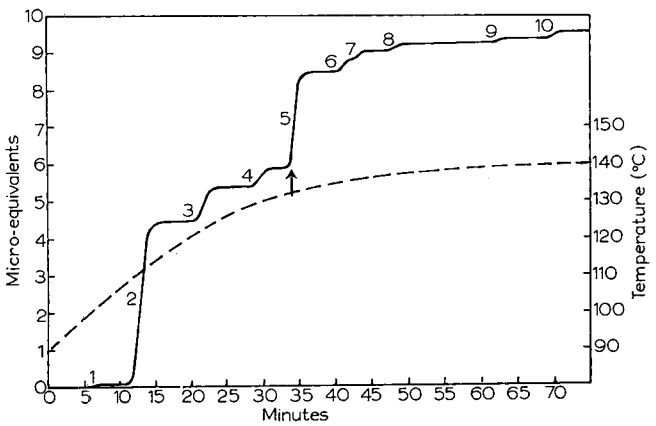


Fig. 3. Gas–liquid chromatogram from sodium salts of volatile acids isolated from a poorly preserved ryegrass silage. Acids: 1 = formic; 2 = acetic; 3 = propionic; 4 = isobutyric; 5 = *n*-butyric; 6 = isovaleric; 7 = α-methylbutyric; 8 = *n*-valeric; 9 = "isocaproic"; 10 = *n*-caproic. ——— alkali consumed; – – – column temperature. Chromatography as in Fig. 2.

ryegrass silage. Ten acids within the range C_1–C_6 have been resolved. The identity of acid No. 9 has not been established, since only the normal isomer of the hexanoic acids has so far been available for examination by this method. Although the difficult pair isovaleric and α-methylbutyric acids have not been separated completely in this analysis, it is apparent that the degree of resolution on the 125 cm column is adequate for most purposes. All other separations are complete. Absence of tailing of formic acid is especially noteworthy.

To date, approximately 150 silage samples, representing several plant species and a wide range of qualities, have been analysed for volatile fatty acids by the method described above. Recoveries have ranged from 96 to 102 % of values obtained by titration of distillates, with a bias towards slightly low values. Incomplete acidification of salts, when they have been allowed to spread while drying in the boat, seems to be the most likely cause of losses. No evidence has yet been found for the presence in silage of volatile acids above C_6 in the series.

Life of stationary phase

The useful life of a behenic acid–phosphoric acid/Chromosorb column has not yet been determined; but more than 100 analyses have been made on the column in current use, without detectable loss in efficiency. Occasional blocking of the capillary

outlet does occur as a result of "bleeding" of the liquid phase. The plugs are, however, readily removed by immersing the capillary in chloroform, without effect on the column's performance.

DISCUSSION

The particular virtues of the method described lie in its simplicity of application to biological materials, and the comparative ease with which accurate quantitative results may be obtained. Although it has only been evaluated for acids from formic to caproic, inclusive, the method would seem to be useful also for higher homologues within the steam volatile group. JAMES AND MARTIN[1] were able to separate saturated monocarboxylic acids up to and including dodecanoic on their silicone/stearic acid columns by prolonged operation at 137°. However, retention volumes with stationary phases containing orthophosphoric acid are about four times greater than with those not containing this acid and, consequently, elution times for higher acids are so long as to render behenic acid–phosphoric acid columns inconvenient for acids containing more than about six carbon atoms. Other stationary phases, *e.g.* polyesters, are preferable when interest lies in the longer chain acids.

The advantage of the pH-stat detector resides in its specificity and ability to yield a direct measurement of acids eluted from a column. No calibration problems arise as in the case of most other detectors. Thus a troublesome feature, which is a common source of errors in quantitative GLC, is eliminated.

ACKNOWLEDGEMENT

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SUMMARY

A stationary phase comprising behenic acid, 20 parts, and phosphoric acid, 4 parts, on Chromosorb W (acid-washed), 100 parts, gives excellent gas–liquid chromatographic separations of C_1–C_6 saturated monocarboxylic acids when wet nitrogen is used as the carrier gas. The tolerance of this column packing for substantial amounts of water vapour in the gas phase has permitted development of a much simplified procedure for the quantitative analysis of volatile fatty acid mixtures isolated as sodium salts. A readily made laboratory chromatograph, using a commercial, glass electrode, auto-titrator as the detector, is described.

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THE DETERMINATION OF PROTEIN MOLECULAR WEIGHTS OF UP TO 225,000 BY GEL-FILTRATION ON A SINGLE COLUMN OF SEPHADEX G-200 AT 25° AND 40°

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INTRODUCTION

The determination of the molecular weights of proteins by means of gel-filtration on cross-linked dextran gels (Sephadex) has been described by a number of authors. WHITAKER[1] obtained an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume, V, to the column void volume, V_0. Using Sephadex G-75 he covered the molecular weight range 13,000–40,200 and with Sephadex G-100 the range 13,000–76,000. WIELAND, DUESBERG AND DETERMANN[2] found a similar correlation on Sephadex G-200 for molecular weights of 13,000–150,000. Using Sephadex G-75 and G-100 ANDREWS[3] also obtained the relationship for molecular weights in the range 3,500–150,000.

The present paper extends the work of WHITAKER[1] up to molecular weights of 225,000 on columns of Sephadex G-200; evidence is provided showing that considerable extension beyond this is possible. Alternative conditions in which the column temperature and buffer ionic strength are increased to minimise possible aggregation effects are also described.

EXPERIMENTAL

Materials

Proteins of known molecular weight. Thyroglobulin; porcine, lyophilized, water soluble, I = 0.9 %. Catalase; *ex* ox-liver, supplied as a suspension. α-Globulins; equine, fraction IV. γ-Globulins; bovine, fraction II. Alcohol dehydrogenase; *ex* yeast, supplied as a suspension. Ovalbumin; *ex* chicken-egg, lyophilized, salt-free. Lysozyme; *ex* egg-white, two times crystallized, lyophilized. Cytochrome c; *ex* horse-heart, Fe = 0.43 %. All these proteins were obtained from L. Light & Co., Ltd. Serum albumin; bovine, crystallized, from Armour Laboratories. Pepsin; crystallized, 1:60,000, from Sigma Chemical Co.

Dextran-gel. Sephadex G-200, Lot No. To. 3016 supplied by Pharmacia, Sweden.

Citrate buffer. A 0.2 *M* solution was prepared by dissolving 21.0 g "Analar", citric acid, $C_6H_8O_7 \cdot H_2O$, in 200 ml *N* NaOH and diluting to 500 ml with distilled water. The pH of this buffer when diluted with an equal volume of water is 5.0 ± 0.1.

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Small differences from this value can be corrected with *N* HCl or *N* NaOH. 0.25 g thymol/l was added as a preservative. The buffer was used undiluted for preparing the ninhydrin reagent, and diluted with an equal volume of water for column development. When used with the column at 40°, NaCl (5 % w/v) was added to the buffer. When used at 40°, the buffers were boiled to remove air and stored in the reservoir under liquid paraffin; this prevents the formation of bubbles within the column.

Methyl cellosolve. Technical methyl cellosolve was shaken with acidified aqueous ferrous sulphate and distilled. The first 5 % and the last 10 % of the distillate were rejected.

Ninhydrin reagent. This was prepared as described by MOORE AND STEIN[4] by dissolving 0.08 g "Analar" stannous chloride, $SnCl_2 \cdot 2\ H_2O$ in 50 ml of the undiluted citrate buffer. This solution was mixed thoroughly with 50 ml methyl cellosolve containing 2 g "Analar" ninhydrin. The solution could be used for three weeks if it was stored under nitrogen and in the dark.

Diluent. The solution was obtained by mixing equal volumes of water and absolute alcohol. Alternatively, re-distilled *n*-propanol could be used instead of the alcohol.

Procedure

Packing of the column. The Sephadex G-200 was suspended in enough buffer to ensure that after swelling the suspension was still sufficiently fluid to allow air-bubbles to escape readily. The gel was allowed to swell for at least 1 day, preferably 3 days, before pouring into the column, otherwise the swelling continued in the column giving very slow flow rates. The swelling was carried out in the buffer used at the column temperature to be employed. Two jacketed columns of 0.9 cm and 2.0 cm diameter and both 110 cm high with sintered glass plates at the bottom were used. The 2 cm diameter column, which gives the higher resolution, was used for most of the present work. The less accurate 0.9 cm diameter column was examined to establish that it could be used in cases where only very small amounts of material are available. The columns were maintained at 25° ± 1° or 40° ± 1°, including during the preparation of the columns. A cork was placed in the column outlet and the suspension of Sephadex poured down the column surface to minimise the formation of bubbles. When a 10 cm layer of the gel particles had settled out, the cork was removed, whereupon more rapid column packing was achieved. The column was kept filled with the suspension; after prolonged settling and passage of buffer (500 ml with the larger column) through the column, a stable level of gel-particles was achieved. The gel was packed to a height of 105–110 cm in both columns.

Preparation of protein solutions. 0.5–10 mg of each protein was dissolved in 1–2 ml of buffer and if necessary filtered. In solutions containing more than one protein the total did not exceed 10 mg/ml. The catalase and alcohol dehydrogenase were supplied as suspensions. The solid was centrifuged off from these, washed with water, re-centrifuged, shaken with the buffer for 30 min, re-centrifuged and 2 ml of the clear solution placed on the column. In the case of catalase the concentration obtainable was very low.

Chromatography. 1–2 ml of the protein solution was run very slowly down the column surface onto the gel. This was to minimise disturbance of the gel; the usual procedure of covering the surface with filter paper was abandoned, as the point at which buffer or protein solution had just sunk into the gel could not be judged accu-

rately. After the solution had sunk into the gel, two 1 ml portions of the buffer were used to wash in any solution adhering to the column, the first being allowed to sink into the gel before the second portion was used. The column was connected to the buffer reservoir and the column developed. A drop-counting fraction-collector was employed and fractions of approximately 1 ml collected. Each fraction had to be weighed as the drop size varied throughout the development, particularly when a protein was emerging. WHITAKER[1] reports an error of only 0.5–1.0 % in the elution volume from this cause; in the present work variations in drop size of as much as 15–20 % have been recorded. A siphon fraction-collector is to be preferred. The number of drops on the drop recorder was used to judge the exact point of addition of the sample to the column. Proteins were added as mixtures when compatible, or singly if not. One protein could be placed on a column following another, provided there was sufficient elution volume between them, and that the molecular weight of the second was not sufficiently great to enable it to catch up the first during elution. As soon as all the material placed on the column had been eluted the column was ready for re-use. The flow rate never exceeded 0.1 ml/min per cm^2 cross-section of column (about 16 ml/h on the large column), slower than any system used by WHITAKER[1], who showed that flow rates of 0.18–0.42 $ml \cdot min^{-1} \cdot cm^{-2}$ were satisfactory and that higher flow rates of 0.62 $ml \cdot min^{-1} \cdot cm^{-2}$ gave low elution volumes.

Colorimetry. 2 ml of the ninhydrin reagent was added to each fraction, the tube capped and heated in a bath of vigorously boiling water for 20 min. The tubes were cooled and the contents diluted with 5 ml of alcohol–water (1:1) and the optical density measured at 570 mμ on a Hilger Uvispek using 0.5 or 2.0 cm cells. An alternative procedure is to measure the U.V. absorption directly on the fractions to give the position of the protein peaks (see ANDREWS[3]). This requires the use of a preservative which does not absorb in the U.V., or the more rapid development of the column in the absence of preservative. In the absence of a preservative the prolonged use of a single column packing may give rise to fungal or bacterial growth. The use of ninhydrin enables one to use a simple colorimeter, and after development select only the tubes in the region of an elution peak for measurement.

Column void volume. The column void volume was checked on the first run on each freshly packed column, and then on alternate runs using thyroglobulin. Thyroglobulin has a molecular weight of 650,000 (see Table I), which is above the exclusion limit for Sephadex G-200; it passes through the column unhindered and its elution volume is therefore equal to the column void volume. Elution volumes were interpolated to the nearest 0.1 ml by triangulation.

RESULTS

Molecular weight of reference proteins

The protein molecular weights used in Fig. 1 and given in Table I are taken from the voluminous literature on the subject. The value selected for any protein, often from a wide range, is that which appears to represent the true value following an examination of the range of values quoted and their methods of determination. Selected literature values are also given in Table I.

The two blood fractions employed are not homogeneous. MCFARLANE[5] has demonstrated that the sedimentation diagrams for the plasma of normal cow, horse

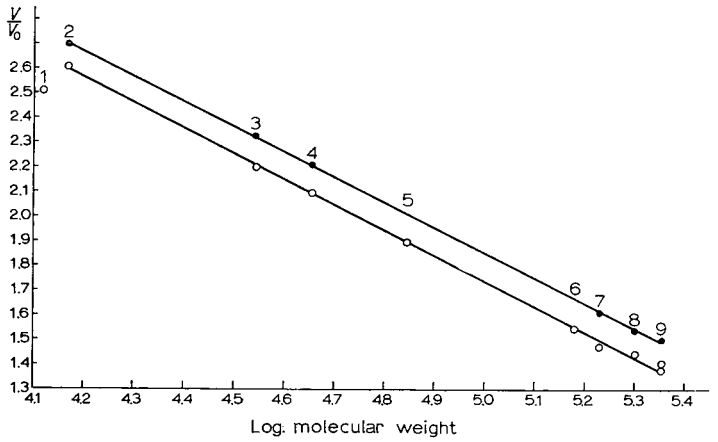

Fig. 1. Relationship between the ratio of the protein elution volume (V) to column void volume (V_0) and the protein molecular weight. ○ 25°, pH 5.0, 0.1 M citrate buffer; ● 40°, pH 5.0, 0.1 M citrate buffer plus 5% NaCl w/v. 1 = cytochrome c; 2 = lysozyme; 3 = pepsin; 4 = ovalbumin; 5 = serum albumin; 6 = alcohol dehydrogenase; 7 = γ-globulin; 8 = α-globulin; 9 = catalase.

and man are qualitatively similar. ONCLEY, SCATCHARD AND BROWN[6] have shown that fraction II of human plasma is predominantly γ-globulin and fraction IV is predominantly α-globulin. It is therefore assumed that fraction II of bovine plasma and fraction IV of equine plasma respectively are of similar composition; the results in Table I support this view.

Void volume

The elution volume of thyroglobulin (mol.wt. = 650,000), which is equal to the void volume for the 0.1 M citrate buffer 25° system, was in the range of 106.3 to 107.0 ml (8 determinations, S.D. ± 0.25 ml) for a column 108.5 cm high and 2.0 cm diameter, measured on alternate runs during a period of 4 weeks. Thus it is necessary to check the void volume of a single column only occasionally.

Effect of column diameter

V/V_0 for α-globulin and lysozyme were 1.440 and 2.603 on the 2 cm diameter column and 1.432 and 2.590 on the 0.9 cm diameter column with the lower temperature system.

Molecular weight and elution volumes

As expected from the earlier work mentioned above, eight of the nine proteins, covering a molecular weight range of 14,600–225,000, gave a linear plot of V/V_0 against log mol. weight. Using the form of equation found by WHITAKER[1], *i.e.*:

$$\text{log mol. wt.} = a\left(\frac{V}{V_0} - 1\right) + b$$

the equation for the lower temperature system was:

$$\text{log mol. wt.} = -0.959\left(\frac{V}{V_0} - 1\right) + 5.700 \qquad (1)$$

TABLE I

MOLECULAR WEIGHTS (mol. wt.) AND THE RATIOS OF ELUTION VOLUME (V) TO COLUMN VOID VOLUME (V_0) OF REFERENCE PROTEINS

System A: Elution at 25° with pH 5.0, c.1 M citrate buffer.
System B: Elution at 40° with pH 5.0, 0.1 M citrate buffer plus 5% NaCl (w/v).

Protein	V/V_0 *in system A*	V/V_0 *in system B*	*Mol. wt. used in Fig. 1*	*Mol. wt. calculated from eqn. (1)*	*Mol. wt. calculated from eqn. (2)*	*Literature values of mol. wt.*	*References*
Thyroglobulin	1	1	650,000			650,000	7
						650,000	8
Catalase	1.377–1.401[a]	1.503	225,000	207,000[a],	225,000	225,000	9
	1.374–1.400	1.488–1.515		228,000		225,000	10
						248,000	11
α-Globulin	1.439[b]	1.547	200,000	191,000	208,000	200,000[c]	6
	1.440	1.525					
γ-Globulin	1.469	1.617	171,000	177,000	177,000	170,000	12
	1.476	1.600				171,000	13
Alcohol dehydrogenase	1.538		151,000	152,000		150,000	14
	1.544					151,000	15
Serum albumin	1.890		70,000	70,000		69,000	16
	1.891					70,300	17
						70,400	18
						71,300	19
Ovalbumin	2.081	2.211	45,000	45,200	45,500	44,400	20
	2.101	2.211				45,000	21
						45,160	22
						45,200	23
Pepsin	2.198	2.318	35,500	35,600	35,500	35,000	24
	2.200	2.322				35,500	25
						35,500	26
Lysozyme	2.616	2.697	14,700	14,600	15,100	14,700	27
	2.590	2.700				14,700	28
						14,800	29
						14,900	28
Cytochrome c	2.508[a, d]		13,200	17,900[a, d]		13,000	30
	2.511					13,400	31

[a] See Results.
[b] Small peak at 1.458.
[c] Human material.
[d] See Discussion.

and for the higher temperature and higher ionic strength system with six of the proteins the equation was:

$$\log \text{mol. wt.} = -0.981 \left(\frac{V}{V_0} - 1\right) + 5.845 \qquad (2)$$

With catalase the peak was very small and flat due to its low solubility in the buffer. To fractions in the region where elution was expected, 10 ml of 1% H_2O_2 was added; the tubes which gave the most rapid evolution of oxygen were in the same position as those detected colorimetrically thus confirming the position of this peak.

For the case of cytochrome c which did not fall on the straight line see Discussion.

Reproducibility

The greatest difference in duplicates of $V/V_0 - 1$ was about 2% for the low temperature system and 4% at the higher temperature. The peaks were broader at the higher temperature.

DISCUSSION

The present work demonstrates that protein molecular weights of at least 225,000 can be determined with Sephadex G-200 with the conditions described. At 25° on a column where the void volume was 106.7 ml, catalase (mol.wt. = 225,000) was eluted at 147.0 ml, indicating that the column can be used in determinations of molecular weights in excess of 225,000.

Cytochrome c behaved as though its molecular weight was 17,900 against literature values of about 13,200. Using acetate buffers WHITAKER[1] found this protein to behave normally. It is of interest to note that this situation was reversed with lysozyme, behaving normally with the present system. It is probably preferable to use the Sephadex G-75 system of WHITAKER[1] for proteins of such low molecular weights, for the slope of V/V_0 *versus* log. mol. wt. is greater. WHITAKER[1] showed that increasing the temperature from 3.3° to 25° resulted in small decreases in V/V_0 and that increases in the ionic strength of the buffer gave slight increases in V/V_0. In the present work the resultant effect of the increase in temperature from 25° to 40° and the addition of 5% NaCl (w/v) to the buffer was an increase in V/V_0. The higher temperature and ionic strength system is designed for the examination of gelatins and their degradation products where aggregation effects must be eliminated.

It should be noted that even if the thyroglobulin was not completely excluded by the Sephadex G-200 the accuracy of the method would not be impaired provided all the measurements for other proteins were made relative to its elution volume for any given column.

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SUMMARY

The present paper describes the use of a cross-linked dextran-gel, Sephadex G-200, for the determination of protein molecular weights of up to 225,000. Evidence is provided which indicates that measurements beyond this are possible. A second system is described, employing a column temperature of 40° and a buffer of high ionic strength which is for use with proteins, such as gelatin, where aggregation effects must be eliminated.

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COLUMN CHROMATOGRAPHIC SEPARATION OF BASIC POLYNUCLEAR AROMATIC COMPOUNDS FROM COMPLEX MIXTURES

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Airborne particulates and other complicated mixtures have been separated through alumina column chromatography, and many polynuclear aromatic hydrocarbons have been determined in the separated fractions through ultraviolet-visible absorption spectrophotometry. Pioneers in this development have been GOULDEN AND TIPLER[1], WEDGWOOD AND COOPER[2], FALK[3] and KOE AND ZECHMEISTER[4]. This method has been applied extensively to the analysis of urban airborne particulates[5,6]. Pentane with increasing amounts of ether has been used as the developing solvent. In this way organic air borne and air pollution source particulates are readily fractionated by one run through an alumina column and then analyzed spectrophotometrically for 9 to 12 polycyclic aromatic hydrocarbons[5].

Another column adsorbent that has been shown to be of value in the separation of polycyclic aromatic hydrocarbons is cellulose acetate[7]. The method has been used extensively in pyrolysis studies[8].

The possible use of π-complexing agents on a column for new types of separations is indicated by the isolation of the benzopyrene fraction minus benzo(*k*)fluoranthene from an airborne particulate sample[9]. Another system that is effective in separating mixtures of polycyclic aromatic hydrocarbons is a liquid–liquid partition chromatographic Fluorisil column consisting of a stationary liquid phase of *sym.*-trinitrobenzene in Carbowax-400 developed by isooctane[10].

Although the use of these various adsorbents for the separation of the more polar aromatic compounds has not been studied, these adsorbent systems should be of value in the analysis of polluted air and other complicated mixtures. In the work described in this paper the same type of alumina column so successful in hydrocarbon analysis[5] has been used with a more polar solvent system for the separation of the basic fraction of complex mixtures into analyzable fractions. Of great value in the characterization and determination of the aza heterocyclic hydrocarbons are the absorption and fluorescence spectra of these compounds in pentane and pentane–1 % trifluoroacetic acid[11].

EXPERIMENTAL

Chemicals and equipment

All solvents were distilled before use. The pure aza compounds were obtained

from commercial sources and were purified by crystallization where evidence of impurity was found.

A Cary recording quartz spectrophotometer Model 11 with 1-cm cells was used for absorption wavelength determinations. Thermo-Vac ovens (Schaar and Company, Chicago, Illinois 60634) were used for careful evaporation of the solutions.

Chromatography

For the chromatographic work Merck acid-washed aluminum oxide was washed with ether, dried, and heated in an oven at 130° for 4 h. The alumina then contained approximately 12 % water (as determined by weighing a sample, heating to a standard red heat for 10 min, cooling in a desiccator, and reweighing). Enough water was then added to the oven-dried alumina to give a final concentration of 13.0 %. (Less water was added for greater resolution; more water for faster separation.) The mixture was shaken and allowed to stand for 12 h in a stoppered container before being used.

One gram of the treated alumina was added to a small volume of the chloroform solution containing 1 to 50 mg of the basic fraction. This fraction was obtained by extracting an organic airborne particulate or air pollution source sample or any other complicated mixture with 10 % aqueous sulfuric acid, neutralizing the acid with sodium carbonate, and extracting this mixture with chloroform. The chloroform on the alumina was evaporated so that the organic material was homogeneously dispersed. This alumina was then added to an 0.5- by 15-in. column, which contained a lower layer of 9 g of the previously prepared alumina and an upper layer of 0.5 g of silica gel, neither containing eluting solvent.

The column was eluted with successive 100 ml volumes of pentane solutions containing the following percentages: of ether—8, 16, 24, 32, 40, 48 and 56, of acetone—5, 10, 15, 20, 25, 30, 35, 40, and then with 100-ml volumes of ether followed by methanol, respectively. Although the column elution required approximately 9 h, most of the known aza compounds were collected before the acetone in pentane solutions were reached; therefore if the operation were stopped at this stage the procedure would involve less than 1 day of operation. The column was protected from light during this period. Fractions of approximately 15 ml were collected and then allowed to stand overnight in the hood.

Spectral analysis

After standing overnight most fractions had evaporated to dryness. The residue in each of these tubes was dissolved in a small volume of pentane and transferred quantitatively by repeated washings to a 3-ml spectral cell of 1-cm light path. The final volume was 3.0 $\pm$ 0.1 ml. The ultraviolet-visible absorption spectrum of each tube was then determined. While these spectra were being obtained, the tubes that still contained eluting solvent were placed in a vacuum oven and carefully evaporated at room temperature in the dark. Then the adsorption spectra of these fractions were also obtained. This procedure can be used with the baseline method for quantitative analysis of the separated basic organic compounds.

For further characterization a drop (about 0.03 ml) of trifluoroacetic acid can be added to the pentane solution in the cell. The absorption spectrum of the salt will then be obtained.

RESULTS AND DISCUSSION

An example of the separation is shown in Fig. 1. Note that the largest molecule comes off the column first. This is because the aza nitrogen is most sterically hindered in this molecule. The alumina adsorbent has a strong attraction for the aza nitrogen. In the figure a dividing line is shown between sterically hindered and non-sterically hindered molecules; this line comes at approximately the thirtieth tube.

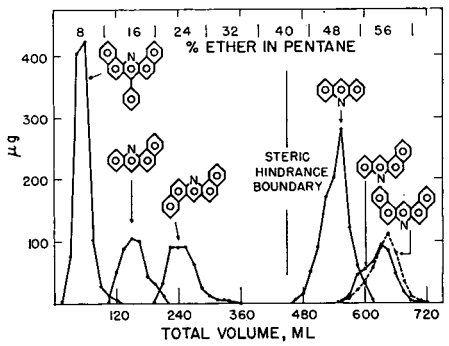

Fig. 1. Elution of 7-phenyldibenz(*c,h*)acridine, benz(*c*)acridine, dibenz(*a,h*)acridine, acridine, benz(*a*)acridine and dibenz(*a,j*)acridine on an alumina column. The three- and six-ring compounds in 1-mg amounts; all others in 0.5-mg amounts.

The dividing line can be moved back further while the elution volume for each compound is increased either by decreasing the percentage of ether in the pentane, by decreasing the amount of water on the alumina, or by lengthening the column. The composition of the mixture chromatographed also affects the position of the dividing line and the elution volumes.

With the described procedure, sterically hindered aza heterocyclic hydrocarbons up to about six or seven rings can be readily separated from the non-sterically hindered aza compounds. Among aza heterocyclic hydrocarbons with the same amount of steric hindrance at an aza nitrogen atom, those with fewer rings are eluted more quickly. Examples of this phenomenon are the pairs: benz(*c*)acridine and dibenz(*a,h*)acridine, acridine and benz(*a*)acridine (Fig. 1). This type of separation is dissimilar from that of the polycyclic aromatic hydrocarbons on alumina, in which the compounds are eluted mainly according to the number of rings in the molecule.

The elution properties of the first compound eluted in Fig. 1 resemble those of the polycyclic hydrocarbons mainly because of the large amount of steric hindrance around the aza nitrogen atom. This resemblance is shown by the fact that polycyclic hydrocarbons containing up to seven fused rings, *e.g.* benzene to coronene, would be eluted in the first six tubes, if they were present.

APPLICATION

The procedure was applied to a complicated mixture, the basic fraction of a sample of coal tar pitch. Coal tar pitch pollution is notorious in that very large concentrations of polynuclear hydrocarbons can be found in extremely high concentra-

tions in the air[12]. The separation on the column worked very nicely. The absorption spectrum in pentane and pentane–1 % trifluoroacetic acid was obtained for each tube. Sharp spectral bands were found in all tubes. It was estimated that sharp bands were obtained for at least 100 basic aromatic compounds. On the basis of position on the column and the absorption spectra in pentane and pentane–1 % trifluoroacetic acid, the following compounds were characterized: an alkylbenzo(*h*)quinoline, benzo(*h*)-quinoline, benzo(*f*)quinoline, acridine, benz(*a*)acridine, and benz(*c*)acridine. Some of the absorption spectra indicated that alkyl derivatives of the benzacridines may be present. The presence of these types of compounds should be more thoroughly investigated, since many alkylated benzacridines are carcinogenic[13]. Examples of the evidence are shown in Figs. 2 and 3. The spectrum of the sixth fraction obtained in this run is very closely similar in pentane (and acidic pentane) to that of benzo(*h*)quinoline (Fig. 2). The unknown compound in pentane solution absorbs 2 mμ further in the visible spectrum, *e.g.*, its long wave-length band is at 348 mμ while the comparable band for benzo(*h*)quinoline is at 346 mμ. From this evidence it is deduced that the

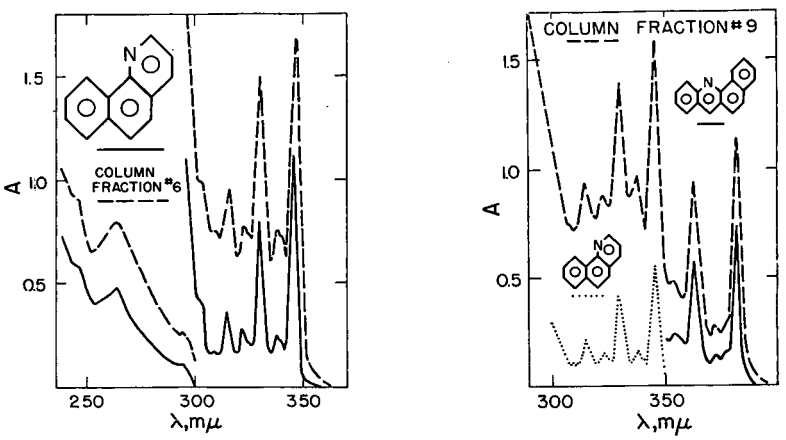

Fig. 2. Ultraviolet absorption spectra in pentane of benzo(*h*)quinoline (———) and subfraction 6 of a basic fraction from coal tar pitch (– – –).

Fig. 3. Ultraviolet absorption spectra in pentane of benzo(*h*)quinoline (· · · ·), benz(*c*)acridine (———) and subfraction 9 of a basic fraction from coal tar pitch(– – –).

compound is an alkyl benzo(*h*)quinoline. A mixture of benzo(*h*)quinoline and benz(*c*)-acridine is found in fraction 9. Some of the evidence is shown in Fig. 3. The bands for benzo(*h*)quinoline and benz(*c*)acridine coincide exactly with the spectral bands found for the fraction. In acidic pentane the fraction gave bands at 294, 332, 410 and 432 mμ; these coincide with the bands obtained for benz(*c*)acridine in the same solvent. In addition the acidic solution of the fraction had bands at 277 (shoulder), 282, 310, 328, 356 and 372 mμ; these coincide with the bands obtained for benzo(*h*)quinoline in the same solvent.

The same type of evidence was used for characterizing the other compounds. The absorption spectrum in pentane solution following column chromatography is the best simple method for characterizing these compounds. The absorption spectrum in acidic solution gives additional evidence, but the spectral fine structure is lost.

CONCLUSIONS

The column chromatographic system introduced in this paper can be used for the quantitative determination of aza heterocyclic hydrocarbons separated from the basic fraction of complex mixtures. Although only six aza heterocyclic hydrocarbons have been characterized in the basic fraction of coal tar pitch, the presence of approximately 100 unknown compounds was deduced from the presence of the many sharp bands in all subfractions. The absorption spectral picture obtained with these fractions is much more complicated than that obtained with the polynuclear aromatic hydrocarbon fractions separated from coal tar pitch.

Many pure aza heterocyclic hydrocarbons must be accumulated before these unknowns can be characterized. Additional means of characterization involving gas, paper, and thin-layer chromatographic methods combined with absorption, fluorescence and phosphorescence spectral methods must be applied before optimum results can be obtained for the routine quantitative determination of the basic polynuclear compounds. In addition, the method should be investigated qualitatively with the basic fractions of various types of urban atmospheric and pollution source particulates before it is applied quantitatively and routinely in air pollution analyses and studies.

SUMMARY

A new method for the column chromatographic separation of basic polycyclic fractions of extremely complicated mixtures is described. Heterocyclic hydrocarbons sterically hindered at the aza nitrogen atom are readily separated from non-sterically hindered aza compounds. Aza heterocyclic hydrocarbons with the same amount of steric hindrance are eluted in the order of their increasing number of fused rings per molecule. The method has been applied to the separation of the basic fraction of coal tar pitch. An alkylbenzo(*h*)quinoline, benzo(*h*)quinoline, benzo(*f*)quinoline, acridine, benz(*a*)acridine and benz(*c*)acridine have been identified. In addition, approximately 100 unknown compounds were believed to be present in the fractions. The evidence for this conclusion was derived from the absorption spectra.

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CHROMATOGRAPHIC ISOLATION AND DETERMINATION OF LONG-CHAIN N-ACYLETHANOLAMINES

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INTRODUCTION

The isolation of N-(2-hydroxyethyl)-palmitamide from soya lecithin, hexane-extracted peanut meal, and hen's egg-yolk was reported by KUEHL *et al.*[1]. Their procedures were not quantitative, and included a saponification step which facilitated the removal of esterified fatty acids but left doubt as to whether this compound actually occurred, as such, in the starting materials. Moreover, it was possible that other N-acylethanolamines ($R \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot OH$) were present but undetected. We have therefore devised quantitative methods for isolating and determining long-chain N-acylethanolamines that are equally applicable to non-saponifiable fractions and to intact lipids from plant and animal sources.

EXPERIMENTAL

Isolation by column chromatography

Silicic acid (Mallinckrodt, A.R.) was activated overnight *in vacuo* at 110° after removal of the largest particles by passage through a 100-mesh sieve and the smallest by sedimentation in methanol (after HIRSCH AND AHRENS[2]). A.R. chloroform was passed through a column of Linde 5A molecular sieve and sampled in a 1 mm infrared cell to verify the absence of hydroxy compounds; 1.00 % (v/v) methanol was added promptly.

A column (d. 19 mm) was prepared from a slurry of 10 g of silicic acid with 40 ml of 1 % methanol in chloroform, and washed until the "veil"[3] was lowered 1 cm. Lipids ($\lesssim$ 250 mg) were then applied, and washing was continued with 250 ml of 1 % methanol. Then 2 % methanol ($\lesssim$ 100 ml) was used to elute N-acylethanolamines, which were recovered by evaporation at $< 40°$.

(The "veil" recedes during development, as the adsorbent withdraws methanol from the eluent, until an equilibrium is established[4]; then the whole column is almost transparent. It is not necessary to reach this stage before lipids are applied. At equilibrium with 1 % methanol in chloroform, Mallinckrodt silicic acid adsorbs about 0.1 ml of methanol per g.)

Thin-layer chromatography

Non-activated layers of Silica Gel G, 0.25 mm in thickness, were used with either (A) benzene–pyridine–acetic acid (40:10:1, v/v/v)[5] or (B) chloroform–acetic

acid–water (180:20:1, v/v/v) as developing solvent. Developed plates were dried at room temperature, lightly sprayed with aqueous NaOCl (13 %, w/w, available Cl), left in a covered dish for 30 min, and then sprayed with ethanol and left exposed for 90 min. Finally, they were sprayed with a mixture (freshly made) of saturated *o*-tolidine in 2 % aqueous acetic acid and an equal volume of 0.05 *N* aqueous KI. N-Acylethanolamines gave blue zones on colourless backgrounds. In solvent A their R_F was about 0.4, and in solvent B it varied between 0.4 and 0.6.

Conversion to N-(2,4-dinitrophenyl) (DNP)-ethanolamine

A solution containing N-acylethanolamines (0.1 μg to 1 mg) was evaporated in a Pyrex test tube (16 × 150 mm) and 1 ml of 6 *N* aqueous HCl was added. The tube was sealed near the top and left overnight in a vertical position in an oven at 108°. When cool it was opened and 1 ml of ether was added. After careful shaking the ether layer was removed by pipette; this washing was repeated twice. Solid $NaHCO_3$ was then added in small excess, followed by 10 mg of 1-fluoro-2,4-dinitrobenzene dissolved in 2.5 ml of ethanol. The tube was closed with a cotton-wool plug and left in a dark cupboard overnight. All subsequent manipulations were carried out with minimum exposure to light. Most of the ethanol was removed in a stream of nitrogen. With 2 ml of water added, the residual mixture was transferred to a separating funnel (PTFE stopcock) and extracted four times with 3 ml portions of chloroform. The combined extracts were evaporated at room temperature, affording an orange residue.

Isolation and measurement of DNP-ethanolamine

The orange residue was chromatographed in ether on a column containing 5 g of activated silicic acid. The first 40 ml of effluent were rejected. The next 40 ml were collected, partly evaporated if necessary, and made up to a suitable volume, and the extinction was measured at 340 mμ. Calculated for N-(2-hydroxyethyl)-palmitamide, $E^{1\%}_{1cm}$ was 450 ± 30.

Confirmation of ethanolamine

HCl hydrolysates were evaporated with warm air and tested for ethanolamine (R_F 0.63) by paper chromatography in *n*-propanol–acetone–0.25 *N* aqueous ammonia (4:1:1, v/v/v)[6].

DNP-ethanolamine (R_F 0.2) was confirmed by thin-layer chromatography in solvent A as described above; samples that had been exposed to light for long periods gave several unidentified yellow zones.

DISCUSSION

Long-chain N-acylethanolamines were found to be eluted from silicic acid columns by 1 % methanol in A.R. chloroform, but retained in A.R. chloroform alone. There was some uncertainty about retention, however, depending upon how much ethanol the manufacturer had added as stabilizer to the chloroform, and so it was preferable to replace the ethanol with methanol at a known concentration. The critical content for elution then became 2 %. (On Florisil the same effect was observed.) Since triglycerides, free sterols, fatty acids, *etc.* were rapidly eluted by 1 % methanol, and most phospholipids and glycolipids by > 2 % methanol, a quantitative and

fairly specific procedure was thus available for isolating N-acylethanolamines occurring in any proportion in any lipid mixture.

The identity of eluted N-acylethanolamines was readily confirmed by infrared analysis (see Fig. 1) and by acidic hydrolysis to fatty acids and ethanolamine. Thin-layer chromatography, employing basic and acidic solvent systems, was useful for

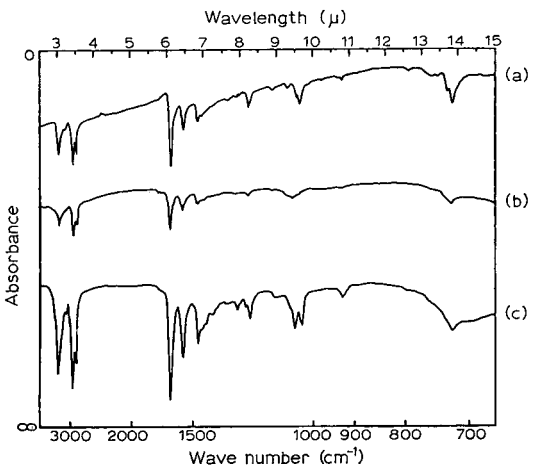

Fig. 1. Infrared spectra of films, cast from chloroform solution, on NaCl plates. (a) Synthetic N-(2-hydroxyethyl)-palmitamide. (b) N-Acylethanolamines obtained after saponification of egg cephalins. (c) N-Acylethanolamines obtained after saponification of corn oil in the presence of ethanolamine.

identification and for detection of N-acylethanolamines in impure eluates. Zones were best revealed by a specific chlorination test for CO·NH, which was adapted from those described by REINDEL AND HOPPE[7] and PAN AND DUTCHER[8]. The limit of detection for N-(2-hydroxyethyl)-palmitamide was 2.5 μg.

For micro-determination, eluted N-acylethanolamines were converted to N-(2,4-dinitrophenyl)-ethanolamine, using essentially the method of AXELROD *et al.*[9]. This derivative was then isolated by column chromatography, in diethyl ether on silicic acid, and measured by spectrophotometry. Excellent separation occurred, provided that the column was not overloaded. In a control experiment, which was designed to test the behaviour of likely impurities, 1-fluoro-2,4-dinitrobenzene (colourless, $\lambda_{max}^{Et_2O} < 228$ mμ), 2,4-dinitrophenol (colourless, $\lambda_{max}^{Et_2O}$ 256 and 330 mμ, $\lambda_{infl}^{Et_2O}$ 281 mμ), and 2,4-dinitraniline (yellow, $\lambda_{max}^{Et_2O}$ 329 mμ) appeared, in order, in the first (rejected) portion of effluent; DNP-ethanolamine (yellow, $\lambda_{max}^{Et_2O}$ 340 mμ) was then eluted in a fairly compact zone. The column separation made this determination considerably more specific than others (for lipid-bound ethanolamine) that are described in the literature[10]. The complete determination was calibrated with synthetic[11] N-(2-hydroxyethyl)-palmitamide.

Milligram quantities of N-acylethanolamines were often determined by direct weighing with, if necessary, correction for any minor impurities revealed by infrared analysis. When major impurities were present the method described for micro-determination was applied.

These methods have facilitated a study of egg-yolk lipids[12] and are now being used in studies of lipids from other sources. Recently UDENFRIEND and co-workers[13,14] used a silicic acid method, which was apparently less specific than that described here, to isolate radioactive N-acylethanolamines, and confirmed their identity by gas–liquid chromatography.

ACKNOWLEDGEMENT

Mr. P. M. LENTHEN collaborated in the early part of this work.

SUMMARY

Elution from silicic acid with 2 % methanol in purified chloroform is the basis of a fairly specific procedure described for the isolation of long-chain N-acylethanolamines from lipid extracts and fractions. Identification and determination procedures are also described, including conversion to DNP-ethanolamine and determination of this derivative by a new method.

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PREPARATION OF ^{3}H-VITAMIN D_3, USING COLUMN AND THIN-LAYER CHROMATOGRAPHY*

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Vitamin D is essential for the optimal absorption of calcium in a number of species and influences the resorption and/or accretion of calcium in bone[1]; an effect on the kidney has also been described[2]. The mechanism of vitamin D action is unknown although various general theories have been proposed[3,4,5]. In order to understand more fully the manner by which this vitamin influences calcium metabolism, it would be of considerable value to determine the localization, distribution and metabolism of vitamin D itself. This type of study has been augmented by the preparation, isolation and purification of radioactive-labeled vitamin D.

Described herein are two procedures that have been used in the preparation of vitamin D_3 labeled with tritium. These were based on techniques previously reported by NORMAN AND DELUCA[6] and PENG[7].

METHOD

The tritium-labeled vitamin D_3, as mentioned above, was prepared by modifications of the procedures described by NORMAN AND DELUCA[6] and by PENG[7]; the first involved tritiation of 7-dehydrocholesterol by the Wilzbach procedure and subsequent conversion to vitamin D_3 and the second was done by tritiating vitamin D_3 directly at low temperatures. Each will be described in turn.

Tritiation of 7-dehydrocholesterol

Five hundred milligrams of 7-dehydrocholesterol were exposed to 3 C of ^{3}H gas at 0° and 255 mm Hg pressure for four days by the Wilzbach procedure[8]**. Following evacuation of excess ^{3}H gas, the product was dissolved in 20 ml of chloroform into which freely exchangeable ^{3}H was incorporated; the ^{3}H-contaminated solvent was then removed by vacuum distillation. The specific activity of the crude preparation was 0.015 mC/mg.

Since preliminary purification entailed losses, the tritiated 7-dehydrocholesterol in the crude preparation was converted directly to vitamin D_3 by ultraviolet irradiation by the following procedure: 20 mg of the crude ^{3}H-7-dehydrocholesterol was dissolved in 40 ml of anhydrous ether and placed in a quartz cuvette. Irradiation was carried out by the use of a sunlamp (LUXOR 20900-2 from Hanovia Lamp Division,

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** Tritiation procedure done by New England Nuclear Corporation, Boston, Mass.

Newark, N.J.) at a distance of 8" with a quartz lens interposed between the cuvette and the lamp. The mixture was constantly stirred with a magnetic stirrer during the process. An irradiation time of 30 min gave maximum vitamin D_3 production. The ether was evaporated under a stream of nitrogen at room temperature. The crude irradiated product was first chromatographed on a water-jacketed, 13 × 100 cm column packed with 35 g of silicic acid (Biorad) slurried in *n*-hexane and under 10 p.s.i. pressure of nitrogen as described by NORMAN AND DELUCA[6]. The sample to be chromatographed was applied to the column in 10% ether in *n*-hexane and eluted with the same solvent at −5°. The eluent was collected in 5 ml fractions delivered at 15 min intervals, and those fractions known to contain vitamin D_3 were immediately refrigerated.

The tritiated vitamin D_3 was identified by the use of thin-layer chromatography (TLC). An aliquot of 100 μl from the 5 ml fractions was spotted on the TLC plates that were coated with 250 μ thick silica gel G and previously activated by heating at 100° for 1 h. All plates used for identification were also spotted with pure 7-dehydrocholesterol and vitamin D_3 for reference purposes. The TLC plates were developed in acetone–*n*-hexane–MeOH (15:135:3) mixture for 50 min, and then air dried and sprayed with a saturated solution of $SbCl_3$ in chloroform for the identification of the steroids. The steroid spots appear at room temperature, but are intensified by blowing a stream of hot air over them. 7-Dehydrocholesterol appears as a green-violet spot changing to intense violet, whereas vitamin D_3 is initially yellow, changing subsequently to brown-orange. The colors are stable for 3 to 4 h but, with time, the spots fade and become hazy in outline.

The fractions now shown to contain vitamin D_3 were pooled and the solvent removed under a stream of nitrogen. The crude vitamin D_3-^3H was then dissolved in chloroform and spotted on the TLC plate to a maximum concentration of 100 μg per spot. Twenty μg of pure, commercial 7-dehydrocholesterol was applied on either side of the vitamin D_3-^3H spots and the plate was developed in the solvent system given above for 50 min. After developing, the plate was air dried and examined under U.V. light. The fluorescing 7-dehydrocholesterol spots were useful in locating the vitamin D_3-^3H regions, since it was previously determined that the vitamin D_3 zone begins immediately above the 7-dehydrocholesterol spot for about 10 to 13 mm. Another fluorescent spot (a degradation product of vitamin D_3) occasionally appears above the vitamin D_3 zone, which further aids in delineating the vitamin D_3 area. Once located, the vitamin D_3 zone was removed by scraping with a sharp spatula. The traces of silicic acid adhering to the glass plate were removed with a cotton swab soaked in chloroform. The vitamin D_3-^3H was then eluted from the silicic acid with chloroform. Recovery by this procedure was found to vary from 85 to 95% as checked previously with known amounts of the vitamin D_3.

The concentration of vitamin D_3-^3H in the preparation was determined by its absorbency at 264 mμ in alcohol (λ = 264, ε = 18,200), using a Beckman DU spectrophotometer. The tritium activity was measured by counting in a liquid scintillation detector (Packard Tricarb) by the usual procedures[9].

The purification of vitamin D_3-^3H on a TLC plate was repeated until a constant specific activity product was obtained and the U.V. fluorescent by-products were removed. The U.V. and the infra-red spectra (Infracord, Perkin-Elmer) of the final purified tritiated product was the same as that for crystalline vitamin D_3.

Usually, a two-fold purification was sufficient, but occasionally it was necessary to re-purify three to four times. During these procedures, it was observed that temperature was an important factor in the stability of vitamin D_3-^{3}H; however, degradation products appear with time even when stored at —20° under nitrogen. This indicates that, for biological experimentation, vitamin D_3-^{3}H should be purified immediately before use. The purification by the present TLC method would be of advantage since the entire operation takes about 2 h.

Direct tritiation of vitamin D_3

Vitamin D_3 was directly tritiated, according to the method of PENG[7,*]. Fifty mg of vitamin D_3 was tritiated with 3 C of ^{3}H gas at —196° and 130 mm Hg pressure for 39 days. The tritium gas was evacuated and the vitamin D_3-^{3}H dissolved in 10 ml of methanol. The contaminated solvent was removed by vacuum distillation and this procedure was repeated. Ten mg samples of the crude vitamin D_3-^{3}H were column chromatographed and the pooled vitamin D_3-^{3}H fractions purified on TLC plates (as previously described) to a constant specific activity and until no by-products were detected by infrared and ultraviolet spectroscopy, or by inspection of the plates under U.V. light for fluorescent areas.

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern of some of the components of the crude tritiated preparation and also illustrates the patterns observed on the TLC plates. It is seen that a separation of the major products (tachysterol, vitamin D_3 and 7-dehydrocholesterol) was obtained on the column and that these could be reasonably separated by TLC. In addition, the separation on the TLC was shown to be facilitated by the addition of methanol to the acetone–hexane solvent; a comparison of the R_F values obtained with the acetone–hexane system with and without methanol is given in Table I. It is apparent that the methanol addition not only increased the

TABLE I

R_F VALUES FOR 7-DEHYDROCHOLESTEROL, VITAMIN D_3 AND THE BY-PRODUCT (PINK COLORED) WITH TWO SOLVENT SYSTEMS[a]

	R_F value	
	n-Hexane–acetone (135:15)	*n-Hexane–acetone–methanol (135:15:3)*
7-Dehydrocholesterol	0.20	0.46
Pink by-product (as colored by $SbCl_3$)	0.23	0.56
Vitamin D_3	0.26	0.58

[a] R_F values represent the mean of 6 runs.

degree of separation between 7-dehydrocholesterol and vitamin D_3, but also separated the former compound from other by-products so that it could be recovered and subsequently converted to vitamin D_3.

* Tritiation procedure done by New England Nuclear Corporation, Boston, Mass.

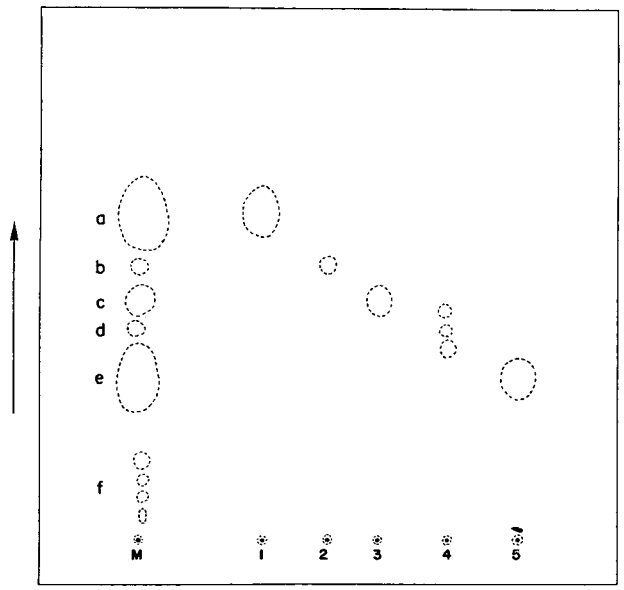

Fig. 1. Identification on TLC plates of compounds from crude U.V.-irradiated 7-dehydrocholesterol-^{3}H as collected from a silicic acid column. Eluting solvent on column: 10% ether in *n*-hexane, temp. —5°. Developing solvent on TLC: acetone–*n*-hexane–MeOH (15:135:3). Vertical spots were visualized by spraying with $SbCl_3$ in chloroform, these being: (a) tachysterol (yellow-brown); (b) unknown by product (blue-green); (c) vitamin D_3-^{3}H (brown); (d) 7-dehydrocholesterol by-product (pink); (e) 7-dehydrocholesterol (violet); (f) several by-products. On the horizontal axis, 100 μl of the following solutions were spotted at the origin: (M) crude material before column chromatography; (1) column fraction No. 40; (2) fraction No. 50; (3) fractions Nos. 56–60; (4) fraction No. 64; (5) fractions Nos. 68–90.

The specific activities and recoveries of vitamin D_3 prepared and purified by several methods are shown in Table II. It may be seen that tritiation of 7-dehydrocholesterol followed by ultraviolet irradiation gave a product of higher specific activity than obtained from direct tritiation of the vitamin D_3 molecule; the percentage recovery of tritiated vitamin D_3 tended to be greater than that for 7-dehydrocholesterol. However, NORMAN AND DELUCA[6], using a somewhat different method for purification, were able to recover approximately 6.6% of their product.

The major problem in the preparation of vitamin D_3-^{3}H was in the separation of a by-product from 7-dehydrocholesterol which appears exactly between the vitamin D_3 and 7-dehydrocholesterol. This shows up as a pink colored spot upon spraying with $SbCl_3$ reagent, but does not yield a colored product with $KMnO_4$ spray (0.2% in 1% Na_2CO_3 w/v) or with H_2SO_4 as used by NORMAN AND DELUCA[10]. Further, it takes about 1 to 2 h to appear on the plate after spraying. The area first appears as a violet spot similar to that of 7-dehydrocholesterol but, with time, turns pink. This by-product has a maximum absorption at 265 mμ in ethanol (similar to vitamin D_3), has no vitamin D_3 biological activity and is eluted from the column after vitamin D_3, as shown in Fig. 1.

Because of the presence of the pink by-product in the tritiated 7-dehydrocholesterol sample, purification could not be affected by thin layer chromatography alone. However, the crude product arising from the direct tritiation of vitamin D_3

TABLE II

SPECIFIC ACTIVITY AND AMOUNT OF VITAMIN D_3-^{3}H RECOVERED AFTER TRITIATION OF 7-DEHYDROCHOLESTEROL AND VITAMIN D_3

Substance tritiated	*Purification procedure*	*Amount of tritiated material purified (mg)*	*Amount of vitamin D_3 recovered (μg)*	*Per cent recovery*	*Specific activity (d.p.m./I.U.)*
7-Dehydrocholesterol*	Column chromatography followed by TLC	20	435	2.2	744
Vitamin D_3**	Column chromatography followed by TLC	10	758	7.6	234
Vitamin D_3**	TLC alone	10	555	5.6	226
Vitamin D_3**	TLC alone	10	378	3.8	279

* By the method of NORMAN AND DELUCA[5].
** By the method of PENG[7].

could be purified by using the TLC alone (Table II) because of the absence of this by-product.

Novel features of the present system are the use of a modified solvent system composed of acetone–*n*-hexane–methanol (15:135:3) that improves the separation of vitamin D_3 and 7-dehydrocholesterol on TLC plates, and the use of saturated $SbCl_3$ in chloroform which detects a contaminated by-product not detected by other procedures. It was also helpful to use reference spots of pure 7-dehydrocholesterol on the TLC plates so that the vitamin D_3 zones could be located under U.V. light without chemical treatment of the plate. During the course of these investigations, it also became apparent that vitamin D_3-^{3}H is highly labile even when stored at —20° under nitrogen. The consequence of this is that the vitamin D_3 products must be re-purified immediately prior to use.

SUMMARY

Vitamin D_3 was prepared by two different methods as reported by others, *i.e.*, (a) the tritiation of 7-dehydrocholesterol by the Wilzbach procedure with subsequent conversion to vitamin D_3 and (b) the direct tritiation of vitamin D_3 at —190° under nitrogen. A combination of column and thin-layer chromatographic procedures were necessary to purify the product resulting from procedure (a), primarily due to the presence of a pink by-product (after $SbCl_3$ treatment) arising from the degradation of 7-dehydrocholesterol. Purification of the product resulting from procedure (b) could be affected on thin-layer chromatographic plates alone. A solvent is described which increases the degree of separation of vitamin D_3 and 7-dehydrocholesterol on TLC plates.

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REPRODUCIBLE R_F VALUES IN THIN-LAYER ADSORPTION CHROMATOGRAPHY

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As commonly practised the technique of thin-layer adsorption chromatography is likely to give very variable results. For this reason many users hesitate to quote R_F values as they would in the case of paper chromatography. Though it is not essential for R_F values to be constant, there are many occasions when reproducibility is a distinct advantage.

The factors affecting R_F values in a given system have been investigated by many workers. TRUTER[1], STAHL[2] and RANDERATH[3] have reviewed most of this work. Further investigations have been recently reported[4–9]. These studies, however, fail to account for a number of variations in R_F values experienced in this laboratory. The subject has therefore been re-examined, in order to develop a practical method of obtaining reproducible R_F values.

TABLE I

AZO COMPOUNDS USED IN THE INVESTIGATION

Abbreviation	*Compound*
AB	*trans*-Azobenzene
DAB	*p*-Dimethylamino-azobenzene*
S3	Sudan III (British Drug Houses Ltd., London)
AAB	*p*-Amino-azobenzene
HAB	*p*-Hydroxy-azobenzene

* Also known as Dimethyl Yellow and as Butter Yellow.

This whole investigation has been carried out almost exclusively with one adsorbent–solute–solvent system; the adsorbent chosen was Kieselgel G (Merck*), since a standard product in common use was desirable; the solute was a mixture of well defined coloured azo-compounds (see Table I) and the solvent was benzene, which is readily available in a high state of purity. Since the work was begun, HONEGGER[4,5] has published results with a very similar system, with which some comparisons can be made.

* Unless otherwise stated, all results were obtained with one batch (No. T 61123).

PRINCIPAL FACTORS STUDIED

Three factors especially have been investigated by the author; these are:

(A) The activity of the adsorbent at the time of development and the effect on activity of the relative humidity of the atmosphere.

(B) The pre-adsorption of solvent vapour by the adsorbent before the liquid phase reaches it during development of the chromatogram.

(C) The variation in the ratio of the liquid to solid phase in the direction of the development.

There has been very little systematic study of these three factors reported in the literature. Recent studies by GEISS *et al.*[7,10] on the effect of relative humidity on alumina-coated plates was not apparently extended to silica gel, but it was sufficient to show that the factor could be important where water-free solvent systems are used, as other workers[4,11] have pointed out.

(A) Adsorbent activity and relative humidity

It is known that the activity of silica gel, like that of alumina, depends very much on its content of loosely bound water[11]. In column chromatography a certain proportion of water is added to fully activated silica gel to obtain adsorbent of the required activity; this is clearly not practical in thin-layer chromatography.

It is not often realised that much of the activity, resulting from heating of a silica gel chromatoplate at 110°, is lost if subsequent spotting is carried out in the normal laboratory atmosphere.

We found that more than half the total amount of moisture adsorbed at equilibrium (in an atmosphere of about 50 % relative humidity) was taken up within about three minutes, and that even breathing on a plate during the spotting process could markedly affect R_F values. The effect of relative humidity was therefore carefully examined in this laboratory as a result of these initial findings.

With fully active plates we found that "tailing" of spots was considerable; this "tailing" is said by KLEIN[12] to be due to an increase in "site energy band width" with increase in activity of the silica. KLEIN found the chromatographic resolving power (on columns) to be optimum at a certain intermediate activity, and that the best way of achieving such activity was by equilibration of the gel with an atmosphere of a definite relative humidity; his findings are applicable to thin-layer chromatography.

With plates equilibrated at humidities much above 60% we observed that "tailing" again became noticeable; this could be due to the amount of water adsorbed being sufficient to fill many of the fine pores and thus to reduce seriously the available surface area of the gel. Over the range 15–58 % humidity no significant variation in tailing was noticed in the particular system studied; on the other hand, there were considerable variations in both R_F values and relative R_F values (see Table II).

We have shown that strict control of the relative humidity, and hence of activity, greatly improves the reproducibility of R_F values. This control is achieved by equilibration of the chromatoplate with an atmosphere of constant relative humidity immediately before development in an S-chamber*. The relative humidities above certain saturated salt solutions[16] are sufficiently constant.

* This was described by E. STAHL[2]. Simple versions have recently been described by DAVIES[13], WASICKY[14] and JÄNCHEN[15].

The results in Table II show how R_F values in benzene vary with the relative humidity. It is clear that precise control of humidity is necessary, if R_F values are to be reproducible to within $\pm$ 0.01 units.

As the activity of the silica gel decreases the separating power decreases (*i.e* R_F values tend to become less different from one another). Hence relative R_F values should also show smaller differences from one another: see Table II. It is concluded that,

TABLE II

VARIATION OF R_F AND R_x VALUES WITH RELATIVE HUMIDITY

Substances, azo-compounds as in Table I; Kieselgel G (Merck), *ca.* 200 μ; "AR" benzene, dried over $CaSO_4$; S-chamber; 10.0 cm; "overrun" 15 min; temperature of humidity chambers = 20°; temperature during developments = 22 $\pm$ 2°. The above results are in most cases the mean of duplicate experiments.

Relative humidity[16]	*Salt solution*	R_F *Values*					R_x *Values**				
		AB	*DAB*	*S3*	*AAB*	*HAB*	*AB*	*DAB*	*S3*	*AAB*	*HAB*
0%	—	0.81	0–0.12	0–0.05	0.05	0.04	—	100	—	—	—
15%	$LiCl \cdot H_2O$	0.90	0.47	0.25	0.13	0.07	191	100	53	27	15
32%	$CaCl_2 \cdot 6H_2O$	0.94	0.62	0.37	0.19	0.09	150	100	59	30	14
52%	$Na_2Cr_2O_7 \cdot 2H_2O$	0.97	0.74	0.57	0.26	0.10	131	100	77	35	13
58%	$NaBr \cdot 2H_2O$	0.974	0.777	0.632	0.329	0.121	125	100	81.0	42.2	15.6
78%	$Na_2S_2O_3 \cdot 5H_2O$	1.00	0.94	0.88	0.58	0.24	106	100	93	61	25
93%	$Na_2SO_4 \cdot 10H_2O$	1.00	1.00	1.00	1.00	0.90	100	100	100	100	90
100%	H_2O	1.00	1.00	1.00	1.00	1.00	100	100	100	100	100

* R_x values = R_F values relative to DAB = 100.

unless the activity is kept constant, there is little advantage in using "markers", provided that the solvent front can be accurately located.

The rate at which the layer of silica gel reaches equilibrium with the moisture of the ambient atmosphere will depend on its thickness, as HONEGGER[5] has pointed out. No systematic study of the time required has yet been carried out. However, HONEGGER's figures suggest that 72 h are necessary for 3 mm layers; for normal 0.25 mm layers our results indicate that 16 h are quite sufficient. The equilibration of plates in constant humidity chambers can be undertaken before spotting (the method is described in the experimental section).

(B) Pre-adsorption of solvent vapour by adsorbent

It was found in this laboratory that the amount of benzene adsorbed by silica gel from air saturated with benzene vapour is a considerable proportion of that required to "wet" the gel. It follows that, if a chromatoplate is equilibrated with a saturated benzene atmosphere before development in liquid benzene, then R_F values should be lower than normal. This is because less mobile phase actually travels up the plate to wet it, part of the necessary benzene being already in the pores of the gel.

When chromatoplates were left in a benzene saturated atmosphere before development, in the manner described by WOLLISH *et al.*[17] and by TRUTER[1], R_F values were found to be fairly reproducible, but were all lower than with development in an S-chamber (see Table III). It was also observed that the R_F values under the two conditions of development bore a nearly constant ratio to one another. The small

TABLE III

EFFECT OF PRE-EQUILIBRATION IN SOLVENT VAPOUR ON R_F VALUES

Substances as in Table I; conditions as for Table II; but relative humidity *ca.* 47 %.

Compound	*On pre-equilibrated plates**		*Normal plates in S-chamber***		*Ratio of R_F values in the 2 systems*§
	R_F values	*R_x values****	*R_F values*	*R_x values*	
DAB	0.500	100	0.712	100	0.702
S3	0.363	72	0.509	71	0.673
AAB	0.170	34	0.258	36	0.659
HAB	0.065	13	0.099	14	0.656

* Mean of 4 experiments; see experimental section for description of the method.

** These R_F values are those estimated from values in Table II for silica gel of the same activity. The assumption is made that, if R_x values are the same, the activity of the silica is the same in the two systems.

*** See Table II.

§ The ratios of HONEGGER's[5] results in his "SN" and "SK" tanks on 250μ layers of the same adsorbent (dried in air 72 h) are similar, 0.708 for Butter Yellow, 0.667 for Sudan Red G and 0.632 for Indophenol. His "SK" tank was similar to the "SN" (normal S-chamber) except that the tank was lined with filter paper soaked in benzene beforehand.

decrease in this ratio with decreasing R_F suggests that a little pre-adsorption of solvent vapour occurs in the S-chamber. With the solvent front moving more slowly as it rises up the plate, there will be more time in the higher regions of the layer for solvent vapour to diffuse ahead of the solvent front. Such a picture fits the observed results in Table III.

With an S-chamber the layer is not initially placed in contact with an atmosphere partly or wholly saturated with solvent vapour; moreover, the volume of the atmosphere in such a chamber is not large in comparison with the volume of adsorbent. Such conditions result in pre-adsorption of solvent vapour being at a minimum. Consequently, in this S-chamber R_F values are not only higher, but are also more reproducible and more nearly "absolute" than those obtained in ordinary tanks.

The adsorption of benzene vapour by a $\sim$ 0.3 mm layer of silica gel under normal chromatographic conditions was measured (see experimental section). Approximately 50 % of the total benzene adsorbed at equilibrium was taken up within 30 min, which is of the same order of duration as a normal chromatogram. It is concluded that the average chromatogram in a simple rectangular tank is developed under conditions under which a variable amount of solvent vapour is being adsorbed by the gel layer. It is most probable that the upper surface of a layer will adsorb faster than the under surface and this fact could help in explaining some conflicting results in the literature with regard to the effect of layer thickness on R_F values.

(C) Ratio of liquid to solid phase on chromatoplates

It is normal practice to allow the solvent to ascend the chromatoplate freely and to remove the plate from the developing chamber after a certain time and before the solvent front reaches the top of the adsorbent layer. When a plate, during development in a hydrocarbon, is viewed by transmitted light it will be seen that the transparency falls off towards the solvent front. This effect has been shown to be due to a decreasing

ratio of liquid to solid phase. It is equally apparent with horizontal as with vertical development.

A developed chromatogram was left in the solvent after the front had reached the top of the adsorbent film; two things were observed. Firstly, the transparency soon became uniform and, secondly, R_F values increased a little (see Table IV). The increase of R_F did not continue once the solvent distribution had become uniform over the plate, unless solvent was allowed to evaporate from the top of the plate. This way of leaving the plate in the solvent after the solvent front has reached the top of the layer will be referred to as "overrunning"; thus it might be said, for instance, that a given chromatogram was "overrun" 15 min.

TABLE IV

EFFECT OF "OVERRUNNING" ON R_F VALUES

Substances as in Table I; same conditions as for Table II, but plates equilibrated 24 h over saturated $NaBr \cdot 2H_2O$ at 20° before use.

Technique	*Solvent front (cm)*	*Total time (min)*	*R_F values*					*R_x values**				
			AB	*DAB*	*S3*	*AAB*	*HAB*	*AB*	*DAB*	*S3*	*AAB*	*HAB*
Normal	10.0	33	0.95	0.72	0.57	0.29	0.11	131	100	79	40	14
Overrun 15 min	10.0	47	0.975	0.775	0.625	0.325	0.11	125	100	80.5	42	15
Normal	13.1	47	0.95	0.71	0.57	0.30	0.10	134	100	79.5	42	14
Overrun 15 min	13.1	69	0.96	0.76	0.61	0.31	0.11	128	100	81	41	15

* See Table II.

This "overrunning" technique has the following advantages:

(a) R_F values across the plate become a little more constant; this makes comparisons with control substances more reliable.

(b) R_F values in single component solvents become independent of the distance from origin to solvent front (see Table IV).

(c) The distance from origin to solvent front is accurately pre-determined. This is important with mixed solvents where frontal analysis of the solvent occurs, resulting in a concentration gradient up the plate. (BRENNER *et al.*[9] have pointed out that, for reproducible R_F values to be obtained under these conditions, the ratio

$$\frac{\text{Distance from "immersion line" to origin}}{\text{Distance from "immersion line" to solvent front}}$$

must be constant.)

(d) The R_F values become more accurately comparable with retention volumes on columns. This follows from the constancy of the ratio of liquid to solid phase up the plate and consequently of the term A_L/A_S in the classical MARTIN AND SYNGE equations.

The time necessary for the ratio of liquid to solid phase to become constant may vary with solvent and distance of travel. (15 min were found to be enough for benzene moving 10 cm). The overrunning must not be extended unnecessarily, since diffusion

of spots continues after the solvent has ceased advancing. A general disadvantage is seen in the fact that the time of development is increased with little, if any, gain in resolution.

This overrunning technique has been incorporated in the general chromatographic procedure employed in this work (see experimental section).

OTHER FACTORS AFFECTING R_F VALUES

In the light of the three main factors discussed above other better known factors were briefly examined:

Liquid–vapour equilibrium in solvent chamber

It is clear from the published literature[2,4,9,15] why this is an important factor. Very good equilibrium is necessary, especially with volatile solvents, and with the S-chamber this is rapidly obtained only where it is needed, *i.e.* in the immediate neighbourhood of the liquid phase on the adsorbent. Because of this and because of the minimum pre-adsorption of solvent vapour, as explained earlier, this type of developing vessel has been used throughout this work, except where it is stated otherwise.

Position of origin and distance of travel of solvent front

This has been included in the discussion of factor C above (see also Table IV).

Temperature

The R_F values at 11° were found to be lower than at 22° (see Table V). It was also noticed that the activity, as measured by relative R_F values, was a little greater at the lower temperature. The results suggest that control of temperature is necessary within a range of about two degrees, in the case of benzene.

TABLE V

EFFECT OF TEMPERATURE ON R_F VALUES

Substances as in Table I; same conditions as for Table II, except for temperature; 2 experiments at each temperature.

Temperature	*R_F values*					*R_x values**				
	AB	*DAB*	*S3*	*AAB*	*HAB*	*AB*	*DAB*	*S3*	*AAB*	*HAB*
21.5°	0.975	0.77	0.62	0.325	0.12	126	100	80	41.5	15
10.5°	0.975	0.745	0.57	0.275	0.10	130	100	76	36.5	13
$\Delta R_F/\Delta t$	0.000	0.0023	0.0045	0.0045	0.0018	—	—	—	—	—

* See Table II.

Layer thickness

This factor has been extensively studied by others. A small increase in R_F with increase of layer thickness over the range 0.25 to 1 mm was noted by PATAKI AND KELLER[8], but confirmed by HONEGGER[5] under certain conditions only. In the light of our present studies it seems that HONEGGER's results in the normal S-chamber ("SN" tank) on plates exposed to the atmosphere for 72 h before use are the most reliable; under these

particular conditions no variation of R_F with layer thickness over the range 0.25 to 3 mm was reported. We have found no variation over the range 0.1–0.5 mm in the few tests we have done here.

Amount of material spotted onto plate

No variation of R_F with amount of material applied was observed for up to only 10 μg per component. With appreciably higher loads R_F values were found to increase a little.

Angle of plane of plate with vertical

The two angles, 10° and 90° to the vertical, were studied. With benzene the results were found to be identical. The time of development was also very similar in each case.

Nature of adsorbent

The structure and uniformity of silica gel will depend upon the method of preparation. The results quoted here, except those in Table VI, were all on one batch of Merck silica gel G. It was therefore pertinent to see if different batches of this adsorbent would

TABLE VI

RESULTS WITH DIFFERENT BATCHES OF KIESELGEL G. (MERCK)

Substances as in Table I; conditions as for Table II, but relative humidity = 58% in each case (24 h over saturated $NaBr \cdot 2H_2O$); temperature = 22 ± 1°. Results for batch T 61123 taken from Table VII. Results for batches T 63272 and 387435 were the averages from 2 experiments.

	T 61123		*T 63272*		*387435*	
	R_F	R_x	R_F	R_x	R_F	R_x
AB	0.974	124.7	0.980	130.5	0.985	123.5
DAB	0.777	100	0.750	100	0.795	100
S3	0.632	81.0	0.590	78	0.665	83
AAB	0.329	42.2	0.315	41.5	0.355	44.5
HAB	0.121	15.6	0.125	16.5	0.135	16.5
Time for 10 cm solvent travel*	33 min		20 min		18 min	

* Without "overrunning" time.

give similar results. Three different batches were examined by the general procedure described here. Fairly small differences in R_F and relative R_F values were observed (see Table VI). A large variation in time of development was, however, noted; this is due to particle size differences. Thus it is clear that the manufacture of silica gel for thin layer chromatography must be carefully standardised, before R_F values can be quoted with real confidence.

STANDARD DEVIATION RESULTS

Table VII gives the results of R_F measurements on 10 chromatograms on one batch of adsorbent. The general procedure described in the experimental section was used; this procedure is designed to minimise the effect of all the variable factors discussed in this report.

TABLE VII

REPRODUCIBILITY OF R_F VALUES IN 10 EXPERIMENTS

Substances as in Table I; same conditions as for Table II, but relative humidity = 58% in each case; temperature = 22 ± 2°.

	AB	*DAB*	*S3*	*AAB*	*HAB*
Mean R_F value	0.974	0.777	0.632	0.329	0.121
Maximum value	0.98	0.79	0.65	0.35	0.14
Minimum value	0.96	0.76	0.62	0.30	0.10
Mid range	0.970	0.775	0.635	0.325	0.120
Standard deviation	0.006	0.010	0.010	0.016	0.013
Mean R_x value*	124.7	100	81.0	42.2	15.6

* See Table II.

The standard deviation (estimated from the range) lay within the limits 0.006 (for $R_F = 0.97$) and 0.016 (for $R_F = 0.33$). It was greater for spots of intermediate R_F value. The temperature variation (± 2°) will account for some of the deviation.

MULTIPLE DEVELOPMENT

Table VIII gives the results of an experiment on double development in one direction. The theoretical R_F values in column 3 were obtained from those in column 1 (1st development) using the equation of STARKA AND HAMPL[18]:

$$R_{F_2} = 2\,R_{F_1} - R_{F_1}^2.$$

The general procedure (see experimental section) was used, and between developments the plate was dried in an oven, and then replaced in the constant humidity tank for 24 h. There was good agreement between experiment and theory.

TABLE VIII

MULTIPLE DEVELOPMENT IN A SINGLE SOLVENT

Substances as in Table I; conditions as for Table II, but relative humidity = 58% in each case (24 h over saturated $NaBr \cdot 2H_2O$). One experiment.

Substance	R_F *value*		
	1st development	*2nd development*	*Calculated*
AB	0.97	1.00	0.999
DAB	0.78	0.95	0.950
S3	0.62	0.86	0.855
AAB	0.32	0.54	0.537
HAB	0.12	0.22	0.221

PREDICTION OF RETENTION VOLUMES ON COLUMNS

When it is necessary to separate macro amounts of material, it is clearly useful to be able to scale up thin-layer chromatograms on to columns. The prediction of retention

volumes on columns, from thin-layer results, will in the first place require accurate R_F values. These values must not only be reproducible, but must also be obtained under conditions that strictly correspond with those in column chromatography. The procedure described here should make it possible to obtain the absolute R_F values required.

A study of the exact relationship between thin-layer R_F values and retention volumes on columns of similar adsorbent is now being undertaken here.

EXPERIMENTAL

A simple method of obtaining plates of reproducible activity was designed. The use of the S-chamber is essential in order to minimise changes of activity of the layer during the development of the chromatogram (*e.g.* by gain by the layer of moisture from the atmosphere of the tank or vice versa). The use of the S-chamber also minimises pre-adsorption of solvent vapour by the layer. Great care must be taken to keep the activity of the plate unchanged after removal of the plate from the constant humidity chamber.

General procedure for obtaining reproducible R_F values

The plates were coated with a layer of Kieselgel G (Merck) approx. 200 μ thick, using a mechanical spreader (Desaga) in the manner described by STAHL[2]. The plates were left to dry in a clean moderately dry atmosphere overnight or longer, and were then prepared for use in the S-chamber by removal of margins of the layer from three sides. The margins were of such width that the three spacing strips of the S-chamber* were separated by several millimeters from the edges of the adsorbent layer. A transverse channel several millimeters wide was also cut from the layer to prevent the solvent front moving beyond this position. Such plates were then left for at least 15 h in a constant humidity vessel maintained at 20°. This vessel was a normal solvent tank (about 7 × 23 × 23 cm), lined with thick filter paper and containing about 50 ml of a saturated aqueous solution of sodium bromide (various saturated salt solutions[16] may be used to obtain different relative humidities, but particularly satisfactory results were obtained with NaBr, which gives 58 % relative humidity, see Table II). Several short lengths of glass tubing in the bottom of the vessel served to keep the plates above the level of the salt solution. For spotting on of the substances a plate was removed from the constant humidity chamber and all adsorbent above the origin immediately covered by a clean glass plate in close contact with the layer. When spotting was complete, the temporary glass cover was quickly replaced by the S-chamber cover plate and the chromatogram immediately developed in the solvent concerned (preferably at 20 ± 1°). The time the solvent reached the top of the layer was noted and the plate left for a further 15 min in the solvent. After development in this way the plate was laid flat and the coverplate removed to allow the solvent to evaporate evenly from the layer.

* The S-chamber used throughout this work was similar to that of DAVIES[14], but, in place of a single piece of glass rod as spacer, three strips of glass, approx. 1.5 × 10 mm in cross-section, were employed. These were stuck to a 20 × 20 cm piece of glass by means of epoxy-resin and ground flat to give a good seal on three sides, when placed over the coated plate.

Pre-equilibration of a plate in solvent vapour

The plate was prepared, spotted and placed in an ordinary tank fitted with a good lid, through which passed the stem of a tap funnel reaching the bottom centre of the tank (see Fig. 1). The tank was lined with thick filter paper and about 25 ml of solvent placed in it: the plate was kept above the level of the solvent by means of a small platform. After 24 h about 100 ml more solvent were added from the funnel to develop the chromatogram without disturbing the equilibrium.

Method of horizontal development

The general procedure, as described above, was used, but development was in a horizontal tank similar to that described by BRENNER *et al.*[19] Great care was taken to reduce solvent vapour loss to a minimum.

Measurement of adsorption of benzene vapour by silica gel

The inside of a wide-neck 100 ml flask was coated with a layer of Kieselgel G *ca.* 0.3 mm thick. The gel was activated 1 h at 110° and weighed *in situ*. The coated flask was placed inside a tank with a saturated benzene atmosphere for a specified time, then re-stoppered and re-weighed. The uptake of benzene was found to reach a maximum of 0.595 ml per g at 20° after about 24 h. This figure is slightly greater than that

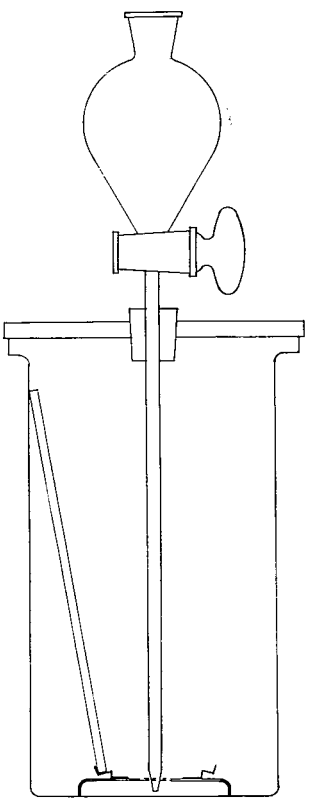

Fig. 1. Tank for pre-equilibration of plate in solvent vapour before development.

for the "total pore volume", 0.54 ml per g, obtained by the direct titration method of MOTTLAU AND FISHER[20]. The small difference could be due to more complete filling of the narrower pores in this vapour adsorption method.

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SUMMARY

The factors affecting the reproducibility of R_F values in adsorption chromatography on thin layers of a given silica gel have been examined. It has been shown that the important factors are method of development and adsorbent activity, which is controlled by ambient relative humidity. Distance of solvent travel and temperature have a moderate effect. Layer thickness and angle of plane of plates appear to have little effect.

A procedure is described for obtaining reproducible R_F values and "absolute" R_F values.

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THIN-LAYER CHROMATOGRAPHIC SEPARATION AND COLORIMETRIC ANALYSIS OF BARLEY OR MALT LIPID CLASSES AND THEIR FATTY ACIDS*

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In the past few years, techniques of thin-layer chromatography have been developed which enable the researcher to separate components of complex lipid mixtures. The technique employs a powdered adsorbent (silica gel "G") affixed rigidly to a glass plate, 15 cm × 15 cm × 0.5 cm[1].

For use in lipid research, two types of thin-layer techniques, adsorption and reverse phase chromatography, have been developed[2]. In adsorption chromatography, a sample is adsorbed on a solid phase of silica acid and a suitable solvent allowed to elute and separate the components. In reverse phase chromatography, a sample is adsorbed in a liquid phase of silicone supported on a silicic acid medium and a suitable solvent is allowed to elute and separate the components. The technique of thin-layer adsorption chromatography has been applied to the fractionation of lipids into their general lipid classes; the technique of reverse phase thin-layer chromatography has been employed for the separation of fatty acids[2]. Used together, adsorption and reverse phase chromatography on thin-layers facilitated detailed analysis of lipid materials.

This paper describes colorimetric procedures for the quantitative estimation of barley or malt lipid classes and their fatty acid composition separated by adsorption and reverse phase thin-layer chromatography.

EXPERIMENTAL

Apparatus and colorimetric procedures

Preparation of lipid extracts

Finely ground barley or malt was extracted with petroleum ether (30–60° b.p.) in a Soxhlet apparatus for a 48 h period. After extraction the petroleum ether solution of lipids was removed and reduced in volume to 10 ml under an atmosphere of nitrogen. The last traces of solvent were removed in a vacuum oven at 20°. All

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samples were then stored under an atmosphere of nitrogen at a temperature of —10° until they were needed for analysis.

Preparation of the plates

Three different types of chromatoplates were used to achieve the separation: thin- and thick-layer adsorption plates, and reverse phase partition plates.

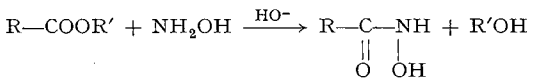
Thin-layer adsorption, used for lipid fractionation, consisted of glass plates (20 cm × 20 cm × 0.4 cm) coated with a thin-layer (250 μ) of silica gel "G". The layer was applied as a slurry consisting of 60 g of Merck silica gel "G" suspended in 100 ml of water and dried in a forced air oven for 2 h at 130°. Thick-layer chromatograms, used for preparative purposes, consisted of glass plates coated with 1000 μ of a silica gel "G".

Reverse phase partition chromatoplates which were used for separation of methyl esters of saturated fatty acids, consisted of glass plates coated with a layer (500 μ) of 10 % siliconized silica gel "G". The gel was applied as a slurry containing silica gel "G" (60 g) suspended in a mixture of diethyl ether (10 ml) and Dow Corning 200 fluid (6 ml). The plate was dried in a forced air oven for 2 h at 130°.

Hydroxamic acid method

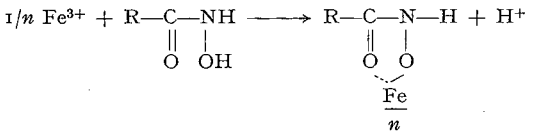
Two colorimetric assay procedures were modified to provide quantitative methods for determination of lipids separated on thin-layer chromatoplates. The hydroxamic acid and the dichromic acid color tests were used.

Lipids that contained ester groups will react quantitatively with hydroxylamine to form hydroxamic acids which form a dark blue chelated complex in the presence of ferric ion. GODDU, LEBLANC AND WRIGHT[3] proposed the following equation for the hydroxamic color reaction:



(1)

Ester + Hydroxylamine ——→ Hydroxamic acid



(2)

(Blue chelated complex)

The following stock solutions, used in the hydroxamic acid reaction, were stored at 5° to minimize degradation:

1. Ferric perchlorate solution. Five grams ferric perchlorate were dissolved in 10 ml of 70 % perchloric acid and 10 ml of water. The solution was diluted to 100 ml with cold 95 % ethanol.

2. Alcoholic NaOH solution. 6 % NaOH in 95 % ethanol.

3. Hydroxylamine solution, 4 % hydroxylamine hydrochloride in 95 % ethanol.

The ferric perchlorate reagent and the hydroxylamine reagent were prepared immediately prior to use. The ferric reagent was prepared by adding 4 ml of the stock ferric perchlorate solution to 3 ml of the 70 % perchloric acid and diluting to 100 ml with 95 % ethanol.

The hydroxylamine reagent was prepared by adding 1 volume of 4 % hydroxylamine hydrochloride in ethanol to 2 volumes of 6 % sodium hydroxide, centrifuging and decanting to remove the precipitated reactant, NaCl.

Lipid samples to be evaluated were removed from thin-layer chromatograms by scraping the marked area of silica gel "G" into a test tube. The silica gel did not interfere with the color reactions, but had to be removed by centrifugation before colorimetric readings could be taken. The colorimeter was adjusted to zero with a blank which contained a similar amount of silica gel "G" that had been scraped from the same chromatogram and treated in the same manner as the sample.

One ml of the hydroxylamine reagent was added to a test tube which contained the lipid to be evaluated. The reagent and sample were mixed thoroughly, heated in a boiling water bath for 1 min, removed from the bath and allowed to cool for several minutes. Three ml of the ferric reagent was added; to remove the silicagel, the reaction mixture was shaken vigorously, centrifuged for 2 min at 3600 r.p.m., decanted into a colorimeter tube, and centrifuged again for 2 min at 3600 r.p.m. The optical density of the mixture was read at 532 mμ in a Spectronic 20 colorimeter. Care was taken to assure that each reading was taken 15 min from the time of the addition of the ferric reagent. The optical density values were then converted to micrograms by the use of standard curves.

Dichromic acid method

The hydroxamic acid colorimetric procedure, specific for ester groups, was not suitable for use with lipids that contained no ester linkages. For these lipids, the dichromic acid colorimetric procedure of JOHNSON[4] was modified and used with thin-layer chromatography.

The dichromic acid reagent, which was stored in a glass stoppered bottle, was prepared by dissolving 5 g of $Na_2Cr_2O_7 \cdot H_2O$ in 20 ml of water and diluting to 1 l with 95 % H_2SO_4.

Lipid samples to be evaluated were scraped from thin-layer chromatograms and contained silica gel "G". The silica gel did not interfere with the color reaction but had to be removed by centrifugation before colorimetric reading could be taken.

One ml of the dichromic acid reagent and 1 ml of water were added to a test tube which contained the silica gel and lipid to be evaluated. The reagent and sample were mixed thoroughly, heated in a boiling water bath for 30 min, removed from the bath and allowed to cool to room temperature. Five ml of water were added; to remove the silica gel, a procedure identical to that used for the hydroxylamine acid test was followed. The optical density of the reaction mixture was read at 440 mμ in a Spectronic 20. The colorimeter was adjusted to an arbitrary optical density of 0.7 with a blank which contained a similar amount of silica gel that had been scraped from the same chromatoplate and treated in the same manner as the sample. The optical density was converted to micrograms by the use of standard curves.

Chromatographic procedures

Lipid fraction preparation

Samples of each lipid fraction were prepared by the use of thick-layer chromatography. The method of MALINS AND MANGOLD[5] was modified for use with 1000 μ thick-layer chromatoplates. The application of lipids to the preparative plate used twenty closely spaced points of application as shown in Fig. 1. About 2.5 mg of lipids were

applied to each of the twenty points; thus, a total of 50 mg was fractionated on each plate.

Thick-layer chromatograms were developed with a solvent system of petroleum ether (60–70° b.p.), diethyl ether, and acetic acid in a volume ratio of 90:10:1.5, respectively[5]. After development, the plates were removed from the chromatographic tanks and dried. The location of the four lipid fractions was determined with short wave ultraviolet light and the areas of the chromatograms which contained the lipid fractions were scraped into sintered glass filters of medium porosity. The lipid fractions were eluted from the silica gel into weighing flasks with 5:1 mixture of chloroform and methanol. Weights of each lipid fraction were determined gravimetrically after removal of the solvent by evaporation in a stream of nitrogen. These lipid fractions were used then to determine colorimetric standard curves.

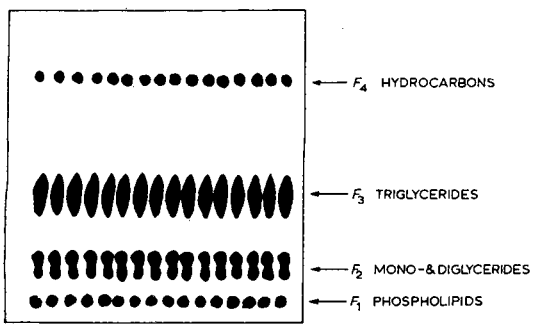


Fig. 1. Thick-layer preparative chromatogram for lipid fractions.

Standard curves

To prepare the standard curves each lipid fraction that was obtained from the thick-layer procedure was rechromatographed by the thin-layer technique in 20 different concentrations (10 μg to 300 μg). After development, the spots were located with short wave ultraviolet light, removed, and placed in test tubes.

The hydroxamic colorimetric method was applied to fractions F_1, F_2, and F_3 while the dichromic acid colorimetric method was applied to fraction F_4, the hydrocarbons. Four standard curves, one for each fraction, were prepared by graphing optical density *versus* micrograms of lipids, one of which is shown in Fig. 2.

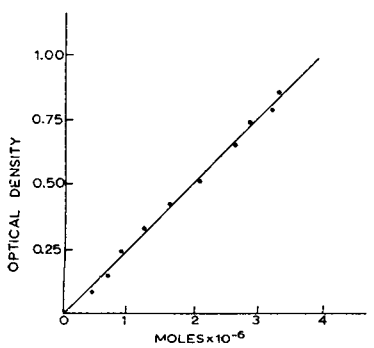


Fig. 2. Standard curves for colorimetric analysis of fatty acid methyl esters.

Inasmuch as all methyl esters or their mercuric acetate adducts gave the same color reaction per micromole[6], a standard curve for methyl esters was determined with methyl oleate (Fig. 2). Pertinent data for all standard curves are contained in Table I.

TABLE I

STANDARD CURVE CORRELATIONS AND REGRESSION EQUATIONS

Standard curve	*Correlation coefficient*	*Regression equation**
F_1 = phospholipids	0.980	$Y = 3.55 \cdot 10^{-3} + 5.84 \cdot 10^{-4} X'$
F_2 = mono, diglycerides	0.982	$Y = 1.61 \cdot 10^{-2} + 3.89 \cdot 10^{-4} X$
F_3 = triglycerides	0.996	$Y = 4.47 \cdot 10^{-4} + 4.2 \cdot 10^{-4} X$
F_4 = hydrocarbons	0.990	$Y = 0.69 - 2.14 \cdot 10^{-3} X$
Methyl oleate	0.985	$Y = 1.80 \cdot 10^{-3} + 0.327 X'$

* Y = optical density; X = micrograms of lipid; X' = micromoles of ester.

Lipid fraction estimation

The extractable lipids of barley and malt were chromatographically fractionated by the method of MALINS AND MANGOLD[5]. This method, as shown in Fig. 3, fractionated the lipids into four broad classes of compounds: the phospholipids (F_1), the mono- and diglycerides (F_2), the triglycerides (F_3), and the hydrocarbons (F_4).

The whole lipids of barley and malt were applied with a micropipet to the thin-layer adsorption chromatoplate about 2 cm from the bottom of the plate. Each chromatoplate was developed with the solvent mixture of petroleum ether (60–70° b.p.), diethyl ether, and acetic acid in a volume ratio of 90:10:1.5, respectively[5].

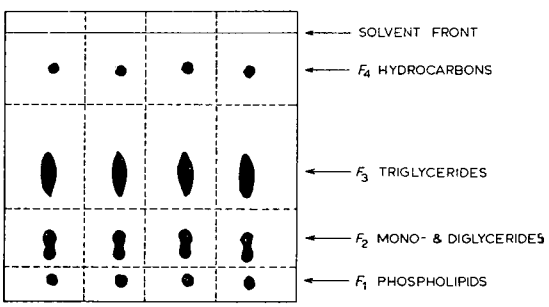


Fig. 3. Thin-layer chromatogram for quantitative analysis of lipid fractions.

The location of the components on the chromatogram was determined by the use of ultraviolet light. No indicator was necessary because each lipid class showed a small amount of fluorescence.

The lipids were removed from the plate by simply scraping into a test tube that area of the chromatogram which contained the desired component. Fractions F_1, F_2,

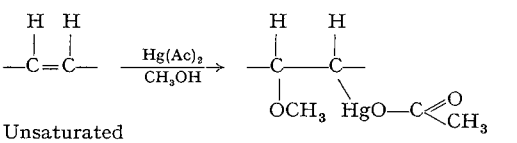
and F_3 were assayed quantitatively by the use of the hydroxamic acid color test for esters[3]. Fraction F_4, containing mostly hydrocarbons, was assayed quantitatively by the use of the dichromic acid color test for organic compounds[4].

Fatty acid analysis

Fifty μg of crude lipids of barley and malt were fractionated by the thick-layer procedure. Each lipid fraction was removed from plates by scraping, placed under 10 ml of methanol, and transesterified to methyl esters by the boron trifluoride method described by METCALFE AND SCHMITZ[7]. After cooling, the methyl esters were removed from the alcohol solution by petroleum ether (30–60° b.p.) extraction and dried with a nitrogen stream.

Due to their polarity, the critical pairs, methyl oleate and methyl palmitate, methyl myristate and methyl linolate, had identical R_F values and could not be separated in this form chromatographically. However, by the use of mercuric acetate addition compounds, the unsaturated esters were separated from the saturated esters[2] in a two step chromatographic procedure to give complete separation of all methyl esters for subsequent colorimetric analysis.

The unsaturated methyl esters were converted to their mercuric acetate addition compounds by heating the ester with 15% mercuric acetate in methanol for 5 min according to the following equation[8]:



Unsaturated ester

Mercuric acetate adduct

The mercuric acetate adducts and saturated methyl ester were removed from the methanol solution by chloroform and water extraction and applied to a thin-layer absorption chromatoplate. At the center and at both edges of the plate, an indicator mixture of mercuric acetate adducts of oleic, linoleic, and linolenic methyl esters was applied. The plate, as shown in Fig. 4, was developed with a solvent system of diethyl ether and petroleum ether (30–60° b.p.) in a ratio of 1:4 v/v to separate the saturated esters from the mercuric adducts. The saturated esters

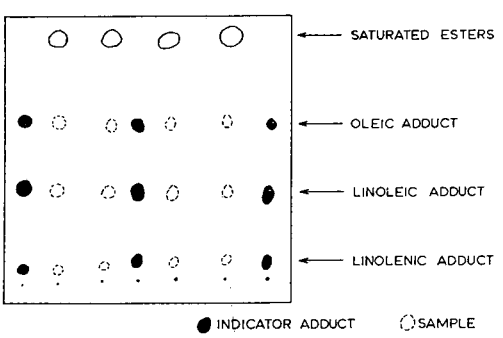


Fig. 4. Thin-layer chromatographic separation of mercuric acetate adducts of unsaturated fatty acids.

were scraped from the solvent front and were reserved for separation on another chromatoplate.

The adsorption chromatogram, which now contained only the mercuric acetate adducts of unsaturated fatty esters, was redeveloped with a solvent system of *n*-propanol and acetic acid (100:1, v/v). The position of each adduct was determined by spraying only the indicator areas with 2,7-dichlorofluorescein, which caused mercuric adducts to become visible as purple spots. Using this as a means of locating the separated adducts of the lipid fractions, the identified areas were scraped into test tubes for colorimetric analysis.

The saturated esters that were removed from the solvent front were placed in an applicator tube (Fig. 5). A methanol:chloroform (1:5, v/v) solution was used to elute the saturated esters from the applicator tube onto a reverse phase siliconized chromatoplate which was fitted subsequently with a 3 inch paper wick (Whatman No. 1) as shown in Fig. 5. The wick provided a means of moving the solvent front beyond

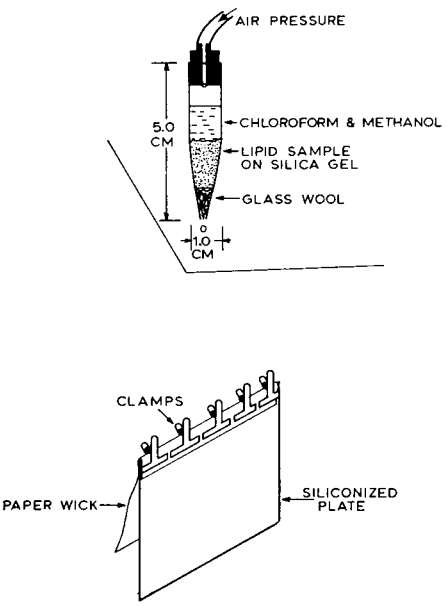


Fig. 5. Applicator tube for transferring saturated esters and siliconized chromatoplate with a paper wick.

silica gel layer thus effecting a better separation of the saturated fatty acid esters. In addition, indicator samples of the unsaturated critical pairs were applied at both edges and the center of the plate and developed with a solvent system of acetonitrile, acetic acid, and water (70:10:25, v/v/v). When the solvent reached the end of the 3 inch wick, the plate was removed and placed in an iodine chamber to indicate the location of the saturated esters by iodine adsorption of the critical pairs (Fig. 6). Each saturated ester was scraped into individual test tubes and quantitatively evaluated by the hydroxamic acid colorimetric procedure.

Optical density readings, taken from the hydroxamic colorimetric procedure,

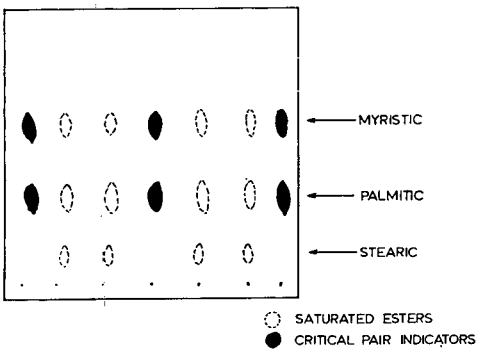


Fig. 6. Reverse phase chromatogram of saturated fatty acid esters.

were converted to micromoles of ester by use of the standard curve determined for methyl oleate (Fig. 2).

DISCUSSION

When used together, the lipid fractionation and fatty acid analytical procedures gave detailed quantitative analysis of lipids. The standard error, based on the colorimetric standard curves for the lipid fractionation method, was $\pm 0.35\,\%$. The standard error, based on the colorimetric standard curves for the fatty acid analytical procedure, was $\pm 0.24\,\%$.

These standard errors indicated that this method was valuable as a tool for quantitative investigation of complex lipid mixtures. Table II shows colorimetric analysis of separated barley lipid fractions. By employing a series of different concentrations, the data show that an application of 1400 to 2200 μg was the most efficient range for evaluation. The lower concentrations were less effective because

TABLE II

RECOVERY OF BARLEY LIPID FRACTIONS DETERMINED COLORIMETRICALLY

Lipid applied (μg)	*Fraction weight*					*Recovery* (%)
	F_1 (μg)	F_2 (μg)	F_3 (μg)	F_4 (μg)	*Total wt. recovered* (μg)	
800	62.44	35.74	522.57	41.99	662.74	82.8
1000	79.57	35.74	713.22	74.62	903.15	90.3
1200	113.83	61.43	832.27	88.61	1096.14	91.3
1400	130.95	87.12	1094.18	111.92	1424.17	101.7
1600	130.95	112.82	1237.03	125.90	1606.70	100.4
1800	148.08	138.51	1427.51	177.18	1891.28	105.1
2000	165.21	164.20	1546.56	191.17	2067.14	103.4
2200	182.34	189.89	1594.18	233.13	2199.54	100.0
				Average		98.8

of the variability caused by lower amounts of lipid contained in fractions 1, 2 and 4. This caused small changes in optical density at the low end of the scale which were difficult to estimate.

Table III further illustrates the precision of the colorimetric determination

TABLE III

RECOVERY OF METHYL OLEATE DETERMINED COLORIMETRICALLY

Methyl oleate applied (μg)	*Methyl oleate determined* (μg)	% *Recovered*
0.6	0.570	95.0
0.8	0.723	90.4
1.0	1.012	101.2
1.2	1.285	107.1
1.4	1.320	94.3
1.6	1.694	105.9
1.8	1.694	94.1
2.0	1.797	89.9
3.0	2.921	97.4
4.0	4.045	101.1
	Average	98.1

for the six fatty acids found in the hydrolyzed lipid fractions. A concentration range of 0.6 to 4.0 μg produced the most accurate results for all six fatty acids. Beyond these limits, the results were more erratic and less dependable.

ACKNOWLEDGEMENTS

The authors wish to thank the Malting Barley Improvement Association of Milwaukee, Wisc. for grants towards the support of this research.

SUMMARY

A method for detailed quantitative analysis of barley and malt lipids was developed. Extractable lipids were fractionated into four broad classes of compounds: phospholipids, mono- and diglycerides, triglycerides and hydrocarbons. The hydroxamic acid colorimetric test for ester groups was modified to quantitatively measure lipids that contained ester groups and the dichromic acid test for organic compounds was modified to quantitatively measure lipids, such as hydrocarbons, which contained no ester group. Standard curves were prepared for the four lipid fractions by the use of a thick-layer preparative procedure. Each lipid fraction can be analyzed for fatty acid composition by a thin-layer chromatographic method which separates fatty acids in the form of saturated fatty acid methyl esters and mercuric acetate adducts of unsaturated fatty acid methyl ester. The method gave quantitative results for both fatty acid analysis and lipid fraction analysis.

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THE ADAPTATION OF A MODIFIED ADAMKIEWICZ-HOPKINS TEST TO THE DETECTION OF INDOLE COMPOUNDS AND THE QUANTITATIVE DETERMINATION OF TRYPTOPHAN BY TLC

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DIAMANTSTEIN AND EHRHARDT[1] and STAHL AND KALDEWEY[2] have detected and identified indole compounds on plates covered with silica gel by using the fluorescence reaction described by PROCHÁZKA[3] and the Ehrlich reaction[4].

We became interested in yet another colour reaction, which is very sensitive and has been used for some time to detect the presence of indole compounds in solution, known as the Adamkiewicz-Hopkins test. This test was modified by FISCHL[5] for use in quantitative estimations of indole compounds in solution. When we used this modified test in our investigation of tryptophan in plasma and blood corpuscles[6], we did not obtain repeatable results.

After further modification of the test we were able to use it for quantitative determinations of indole compounds in biological material[7] and this reaction has now been adapted for use on plates covered with silica gel and powdered cellulose. This test could not be used for paper chromatography because of the destructive power of the concentrated acids in the reagent.

EXPERIMENTAL

Materials

The materials used and their sources are listed below:

(1) DL-Tryptophan (B.D.H.).
(2) Serotonin, creatine sulphate complex (Merck).
(3) DL-5-Hydroxytryptophan (Fluka A.G.).
(4) (Indole-3)-acetic acid (Merck).
(5) 5-Hydroxyindoleacetic acid (manufacturer unknown).
(6) (Indole-3)-propionic acid (B.D.H.).*

METHOD

In our investigations we employed thin-layer chromatography (TLC) with silica gel G 22 and powdered cellulose MN 300 as sorbents. The development was carried out on plates 20 × 20 cm covered with sorbent by means of the spreader made by Desaga. The thickness of the layer of sorbent was 0.3 mm. The separation was carried out in 10 cm sections.

* The numbering of the indole compounds corresponds to that given in Figs. 1–6.

The two solvents used for development were: isopropanol–ammonia 25 %–water (20:1:2) and *n*-butanol–glacial acetic acid–water (15:3:5). Identification was by either the fluorescence method in U.V. light, Ehrlich's reagent (10 % *p*-dimethylaminobenzaldehyde in concentrated HCl–acetone, 1:4), or the modified Adamkiewicz-Hopkins reagent (acetic acid containing 56 mg Fe/l, concentrated H_2SO_4).

The quantitative estimations were made by means of a Pulfrich photometer with an Elpho attachment, in containers 0.5 cm long and an S 53 filter.

RESULTS

The separation of indole compounds on plates covered with silica gel and cellulose was compared; the amount of standard substance deposited was 3 μg. The plates covered with cellulose were activated at 105° for 15 min, while the plates with silica gel were not activated but dried at 20° for 12 h. For results see Figs. 1 and 2.

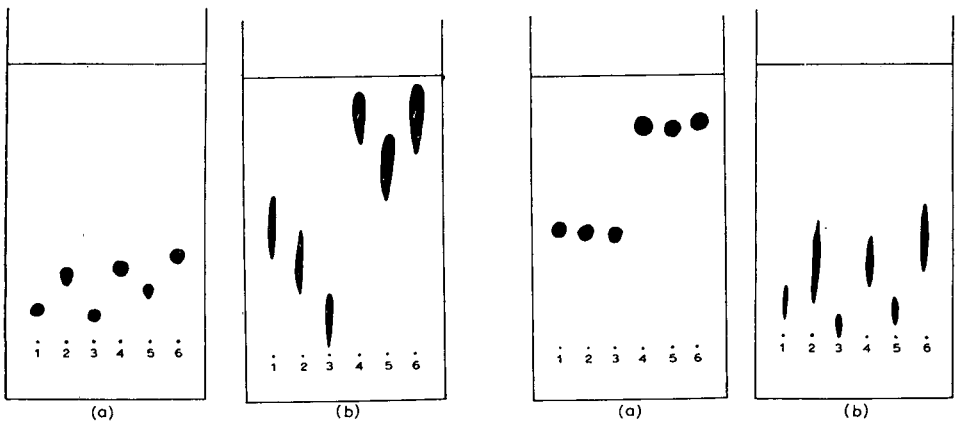

Fig. 1a. Thin-layer chromatogram of indole derivatives on silica gel G 22 with butanol–HAc–water (15:3:5) as solvent.

Fig. 1b. Thin-layer chromatogram of indole derivatives on cellulose MN 300 with butanol–HAc–water (15:3:5) as solvent.

Fig. 2a. Thin-layer chromatogram of indole derivatives on silica gel G 22 with isopropanol–ammonia 25 %–water (20:1:2) as solvent.

Fig. 2b. Thin-layer chromatogram of indole derivatives on cellulose MN 300 with isopropanol–ammonia 25 %–water (20:1:2) as solvent.

Some separations by a two-dimensional technique were also carried out. The following solvent systems were used:

(A) I — chloroform–glacial acetic acid (95:5)
II — methyl acetate–isopropanol–ammonia 25 % (45:35:20)

(B) I — methyl acetate–isopropanol–ammonia 25 % (45:35:20)
II — chloroform–methanol–glacial acetic acid (75:20:5)

(C) I* — isopropanol–ammonia 25 %–water (20:1:2)
II* — *n*-butanol–glacial acetic acid–water (15:3:5).

* The solvents used by us.

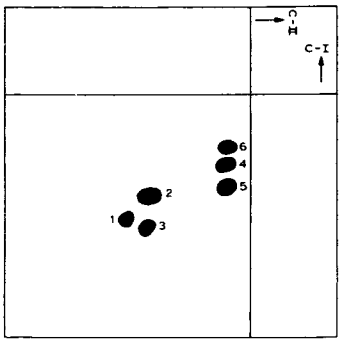

Fig. 3. Thin-layer two-dimensional chromatogram of indole derivatives mixture on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

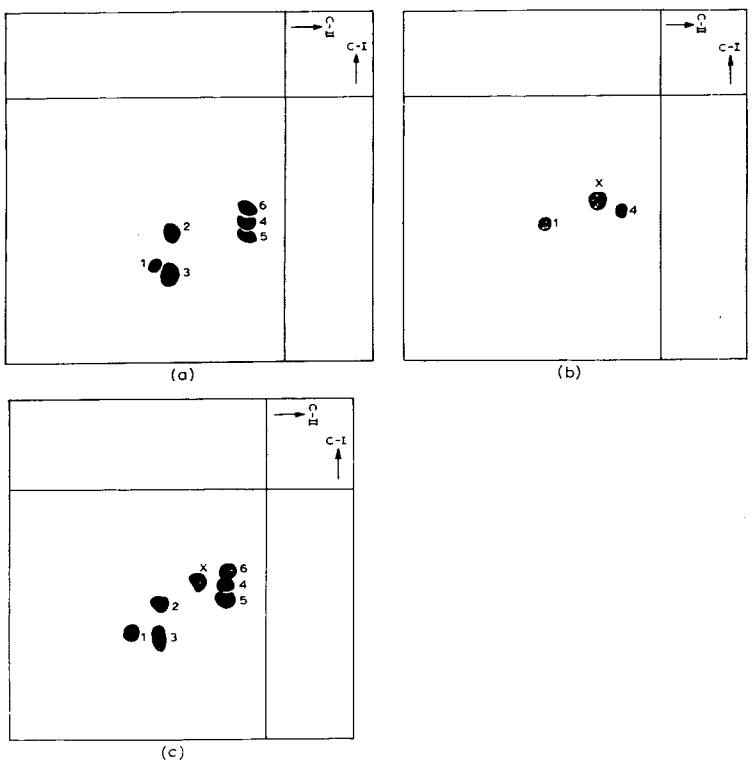

Fig. 4a. Thin-layer two-dimensional chromatogram of indole derivatives mixture on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

Fig. 4b. Thin-layer two-dimensional chromatogram of indole derivatives in urine on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc-water (15:3:5) (II) as solvents.

Fig. 4c. Thin-layer two-dimensional chromatogram of urine with added indole derivatives on silica gel G 22 using isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

TABLE I

COMPARISON OF THE SENSITIVITY OF INDOLE DERIVATIVES TO SPECIFIC COLOUR TESTS ON SILICA GEL AND CELLULOSE

Indole derivatives	*Silica gel G 22*				*Cellulose MN 300*			
	Ehrlich test		*Modified Adamkiewicz-Hopkins test*		*Ehrlich test*		*Modified Adamkiewicz-Hopkins test*	
	Sensitivity (μg)	*Colour*	*Sensitivity* (μg)	*Colour*	*Sensitivity* (μg)	*Colour*	*Sensitivity* (μg)	*Colour*
Tryptophan	0.03	pink-violet	0.05	yellow-violet	0.02	violet	0.03	violet
Serotonin-creatine sulphate	0.04	grey-blue	0.03	yellow-blue	0.01	violet-blue	0.03	violet
5-Hydroxy-tryptophan	0.03	violet-blue	0.03	yellow-blue	0.02	violet-blue	0.03	violet-blue
3-Indolylacetic acid	0.02	violet	0.01	violet	0.01	violet	0.01	violet
5-Hydroxy-indolyl-acetic acid	0.02	blue	0.03	blue	0.02	violet-grey	0.03	violet-grey
3-Indolylpropionic acid	0.03	violet	0.01	grey-violet	0.01	violet	0.03	violet-pink

The separation employing solvent system C is shown in Fig. 3.

The sensitivity of various indole derivatives, on silica gel and on cellulose, subjected to the Ehrlich test and to the Adamkiewicz-Hopkins test, is given in Table I.

Table II shows the R_F values of various indole derivatives separated on silica gel. A comparison is made between the separation obtained in isopropanol–ammonia 25 %–water (20:1:2) and *n*-butanol–acetic acid–water (15:3:5).

Two-dimensional chromatograms of indole derivatives on silica gel and of indole compounds in urine and plasma are shown in Figs. 4 and 5. The detection of indole compounds in the presence of amino acids is shown in Fig. 6.

TABLE II

R_F VALUES OF INDOLE DERIVATIVES ON SILICA GEL G 22

Indole derivatives	*Solvents*	
	Isopropanol–ammonia–water (20:1:2)	*n-Butanol–acetic acid–water* (15:3:5)
Tryptophan	0.11	0.43
Serotonin-creatine sulphate	0.23	0.42
Hydroxy-tryptophan	0.08	0.38
3-Indolylacetic acid	0.26	0.80
5-Hydroxyindolylacetic acid	0.18	0.78
3-Indolylpropionic acid	0.31	0.82

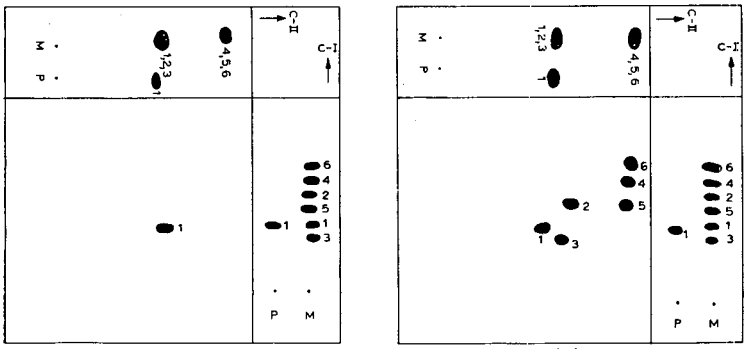

Fig. 5a. Thin-layer two-dimensional chromatogram of human plasma on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents. P = plasma; M = indole derivatives mixture.

Fig. 5b. Thin-layer two-dimensional chromatogram of human plasma with added indole derivatives on silica gel G 22 using isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents. P = plasma; M = indole derivatives mixture.

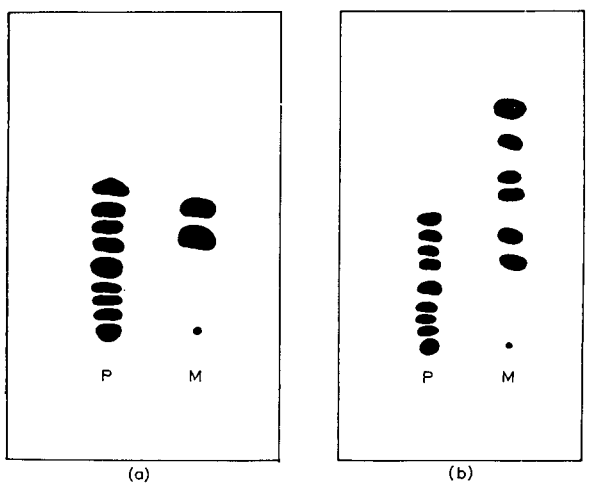

Fig. 6a. Thin-layer one-dimensional chromatogram of amino acids (P) and indole derivatives mixture (M) on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) as solvent. Ninhydrin detection only.

Fig. 6b. Thin-layer one-dimensional chromatogram of amino acids (P) and indole derivatives mixture (M) on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) as solvent. Ninhydrin and Ehrlich tests.

Quantitative estimation

After carrying out the separation of the mixture of indole compounds on plates covered with silica gel or cellulose, and identifying tryptophan by the fluorescence test, the gel corresponding to the tryptophan spot is scraped off the plate. It is eluted in 0.5 ml of water, 1.5 ml of glacial acetic acid containing 56 mg Fe/l and 1 ml of concentrated sulphuric acid. After mixing, the liquid is centrifuged and the tryptophan is estimated in the resulting supernatant. The extinction value for tryptophan

is calculated from the standard curve (see Fig. 7). The loss of tryptophan during elution and the development of the chromatograms was determined as shown in Table III.

TABLE III

RECOVERY OF TRYPTOPHAN AFTER TLC

Tryptophan (μg)	*Without development*		*n-Butanol–acetic acid–water (4:1:1)*			
			Silica gel G 22		*Cellulose MN 300*	
	(μg)	(%)	(μg)	(%)	(μg)	(%)
5	3.5– 4.5	70–90	2.5– 4.0	50–80	2.5– 4.0	50–80
10	6.5– 9.5	65–95	5.5– 9.0	55–80	8.5– 9.0	85–90
15	14.0–14.5	90–97	7.5–10.0	50–61	12.0–15.0	80–100
20	17.5–20.0	87–100	12.0–15.0	60–75	17.0–20.0	90–100
25	22.5–25.0	90–100	15.0–18.0	60–72	23.0–24.5	90–95
30	26.0–28.0	87–90	17.5–20.0	58–70	26.0–27.0	87–90
40	30.0–38.0	80–90	25.0–28.5	62–71	33.0–35.0	82–88

DISCUSSION

The separation of the indole compounds was carried out by thin-layer chromatography using silica gel (Merck) and powdered cellulose without plaster of Paris (Macherey-Nagel MN 300) as sorbents. A better separations was obtained on silica gel; the spots of the indole compounds are compact, clear and well-defined, while on cellulose they run into one another and are indistinct.

In the development of two-dimensional chromatograms we used the solvent systems A, B and C*. System C as used by STOWE AND THIMANN[8] was found to be the best for the substances under investigation. For one-dimensional chromatography the best separation was obtained using solution C I.

Comparing the colour tests of Ehrlich and Adamkiewicz-Hopkins, we found slight differences in their sensitivity. The deviations were in the range of 0.01–0.03. The modified Adamkiewicz-Hopkins test is more suitable for the identification of indole compounds, because the colour remains longer in comparison with the Ehrlich test. When the Ehrlich test is used for the detection of indole compounds in urine, urea gives a diffuse yellow spot; the Adamkiewicz-Hopkins test gives no colouring with urea. The sensitivities of these tests are similar, but that of the fluorescence tests is ten times greater.

Good results in the separation of indole compounds in urine are only obtained in 30 μl without desalting and in 20 μl of deproteinized plasma.

Development of the chromatograms with ninhydrin followed by the Ehrlich test enables the identification of indole compounds in the presence of amino acids to be carried out.

In the quantitative estimations of tryptophan from the plates without development the losses were within the range of 10–30 μg. During the development of the chromatograms in the butanol system, the losses of tryptophan from the gel plates

* The solvents used by us.

were about 40 %, while on cellulose they are not more than 20 %. Probably the greater losses on the gel are caused by the adsorption of the tryptophan on the plaster of Paris, which is a constituent of the gel.

From this investigation it can be seen that the Adamkiewicz-Hopkins test is equally good for the purposes of identification and quantitative estimation.

Attempts at quantitative estimations of other indole compounds are in progress.

SUMMARY

Indole compounds were separated by TLC on silica gel and cellulose and identified by the colour tests of Ehrlich and Adamkiewicz-Hopkins. The tests were carried out on standard substances and on biological materials containing indole compounds.

The modified Adamkiewicz-Hopkins test was adapted for the identification and quantitative estimation of indole compounds.

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THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES IN THE PRESENCE OF BISULFITE

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INTRODUCTION

In the course of studies on the rapid quantitative determination of lactulose (4-O-β-galactopyranosyl-D-glucose) in milk products which are under way in this laboratory, it was necessary to devise a thin-layer chromatography method capable of separating and identifying tagatose, lactulose and sucrose. During the past few years a considerable amount of work has been reported in the literature on the thin-layer chromatographic analysis of carbohydrates[1–7]. However, it was found in a preliminary experiment that the three carbohydrates described above are not well separated by these methods. As a means of eliminating the disadvantages, the chromatographic development in the presence of bisulfite as reported in previous papers[8,9], was investigated for its applicability to thin-layer chromatography.

The present paper describes the adaptation of the principles of chromatographic development in the presence of bisulfite to the thin-layer chromatographic separation of carbohydrates. Clear separation is achieved by development on a chromatoplate of silica gel G mixed with a small amount of bisulfite. After the development the carbohydrates are detected by four different spray reagents: *o*-aminodiphenyl–orthophosphoric acid, carbazole–sulfuric acid, phenol–sulfuric acid and thymol–sulfuric acid. Keto-sugars are selectively detected by a modification of procedure of ADACHI[10].

EXPERIMENTAL

Preparation of the chromatoplates

A slurry of 40 g of "Kieselgel (silica gel) nach Stahl" (E. Merck, A.G., Darmstadt, Germany) in 80 ml of 0.1 *M* sodium bisulfite solution was applied to the glass plates (20 cm × 20 cm × 0.3 cm) at a thickness of about 0.25 mm, using a Toyo Kagaku Sangyo Co. Model HC-20 Spreader. These plates were allowed to stand for 30 min at room temperature, and then dried in an oven at 110° to 120° for 1 h. The coated chromatoplates were cooled and used for chromatography as described.

Development of the chromatoplates

An aliquot containing 5 to 10 μg of pure carbohydrates and of their mixtures in water was applied to the plates with a micropipette in the conventional manner. The plates were developed by the ascending technique, without prior equilibration,

until the solvent front had reached a distance of 13 cm past the spotting position. The following solvent systems were used:

Solvent A: Ethyl acetate–acetic acid–methanol–water (6.0:1.5:1.5:1.0).

Solvent B: Isopropanol–ethyl acetate–water (7:1:2).

Solvent C: Methyl ethyl ketone–acetic acid–water (3:0.5:1.5).

Solvent D: Propanol–water (8.5:1.5).

When the solvent had reached the limiting line, the plates were removed and air-dried in a horizontal position.

Detection of the spots

After the application of the spray reagent, the carbohydrates were detected on the chromatoplates by heating in an oven for the time required by the reagent used.

(1) *o-Aminodiphenyl–orthophosphoric acid reagent.* The chromatoplates were sprayed with a solution containing 0.3 g of *o*-aminodiphenyl and 5 ml of orthophosphoric acid (sp. gr. 1.88 at 20°) in 95 ml of ethyl alcohol. The plates were then heated in the oven for 15 to 20 min at 110°. The carbohydrates appeared as brown spots.

(2) *Carbazole–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 0.5 g of carbazole and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol. After heating the plates for 10 min at 120°, the carbohydrates appeared as violet spots on a blue background. Freshly prepared reagent is preferable.

(3) *Dimedone–orthophosphoric acid reagent*[10]. When the chromatoplates sprayed with this reagent were heated for 15 to 20 min at 110°, ketose-containing carbohydrates appeared as dark greenish grey spots.

(4) *Phenol–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 3 g of phenol and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol, and then heated for 10 to 15 min at 110°. The carbohydrates appeared as brown spots which could be intensified by further heating. The reagent could still be used when several days old.

(5) *Thymol–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 0.5 g of thymol and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol. After heating for 15 to 20 min at 120°, most carbohydrates appeared as dark pink spots on a white background, changing to faint violet on further heating.

RESULTS AND DISCUSSION

The separations of the carbohydrates are shown in Table I together with the colors developed after the application of the spray reagents. Eighteen solvent systems were tested on the chromatoplates using an eight-component mixture of fructose, glucose, lactose, lactulose, maltose, rhamnose, sucrose and xylose as the sample, but on the basis of separation efficiency, good separations of these carbohydrates were only obtained with the solvent systems given in Table I. Solvent D gave the best separation, though some of the carbohydrates were no longer resolved under these conditions. Fig. 1 shows a typical chromatogram developed with the solvent D. Addition of increasing amounts of acetone to solvent D increases the speed of the development, but also increases the migration distance, and changes the shape of the spots from an exaggerated oval type to a cucumber type with the tail of the cucumber towards the origin. Slight variation in migration was occasionally noted from plate to

TABLE I

R_F VALUES AND SPOT COLORS OF CARBOHYDRATES

Carbohydrate	*R_F in solvent**				*Color with reagent***				
	A	*B*	*C*	*D*	*I*	*II*	*III*	*IV*	*V*
L-Arabinose	0.32	0.63	0.51	0.51	B	V	None	DB	PDC
D-Lyxose	0.46	0.68	0.61	0.59	B	V	None	DB	PDC
D-Ribose	0.50	0.69	0.61	0.57	B	V	None	DB	PDC
D-Xylose	0.34	0.68	0.50	0.59	B	V	None	DB	PDC
L-Fucose	0.49	0.62	0.66	0.55	B	V	None	B	PDC
L-Rhamnose	0.57	0.68	0.53	0.62	B	V	None	YB	B
D-Galactose	0.32	0.53	0.47	0.39	B	V	None	B	P
D-Glucose	0.28	0.61	0.47	0.48	B	V	None	B	P
D-Mannose	0.41	0.60	0.57	0.53	B	V	None	B	P
D-Fructose	0.28	0.57	0.53	0.48	YB	V	DGG	GB	PDC
L-Sorbose	0.43	0.56	0.58	0.47	YB	V	DGG	GB	PDC
D-Tagatose	0.46	0.61	0.58	0.53	YB	V	DGG	GB	PDC
2-Deoxy-D-glucose	0.68	0.79	0.75	0.73	DB	V	DP	DB	DG
Lactose	0.08	0.36	0.21	0.23	B	V	None	B	P
Lactulose	0.10	0.40	0.24	0.27	YB	V	DGG	GB	PDC
Maltose	0.11	0.50	0.22	0.35	B	V	None	B	P
Sucrose	0.20	0.55	0.28	0.40	YB	V	DGG	GB	PDC
Trehalose	0.05	0.38	0.08	0.23	None	V	None	B	P
Melezitose	0.10	0.49	0.16	0.30	B	V	None	GB	P
Raffinose	0.04	0.28	0.10	0.13	B	V	None	GB	P

* A = ethyl acetate–acetic acid–methanol–water (6.0:1.5:1.5:1.0); B = isopropanol–ethyl acetate–water (7:1:2); C = methyl ethyl ketone–acetic acid–water (3.0:0.5:1.5); D = propanol–water (8.5:1.5).

** B = brown; DB = dark brown; DGG = dark greenish grey; DG = dark grey; DP = dark pink; GB = greenish brown; P = pink; PDC = pink with dark center; V = violet; YB = yellowish brown. I = *o*-Aminodiphenyl–orthophosphoric acid reagent; II = carbazole–sulfuric acid reagent; III = dimedone–orthophosphoric acid reagent; IV = phenol–sulfuric acid reagent; V = Thymol–sulfuric acid reagent.

plate with the four different solvent systems, thus making it necessary to chromatograph known compounds simultaneously with unknown. STAHL AND KALTENBACH[1] reported that they had been able to separate ribose and xylose on silica gel G plates in the presence of sodium acetate, using the solvent system ethyl acetate–isopropanol (65:35). This system, however, did not give sufficiently good separation of the two pentoses on the chromatoplates described in this paper.

Since the nature of the reactions involved in the separation of the carbohydrates on the thin-layer chromatoplate cannot be presented with assurance, the theoretical implications of these results have not been fully explored. However, it is of interest to point out that such a separation is consistent with the present theory of sugar-bisulfite addition compounds[11,12]. According to the data of INGLES[11] and BRAVERMAN[13], concentrated mixtures of aldo-sugars and an alkaline metal bisulfite in water should give addition compounds at room temperature. Although addition compounds of keto-sugars and bisulfite have not been isolated, it has been suggested that keto-sugars give unstable addition compounds on a strong anion exchanger in the bisulfite form at a high ethyl alcohol or propanol concentration[8,14]. In the case of the separation of carbohydrates presented here, the conditions for the formation of such addition compounds are fulfilled, and thus it seems reasonable to assume that definite equili-

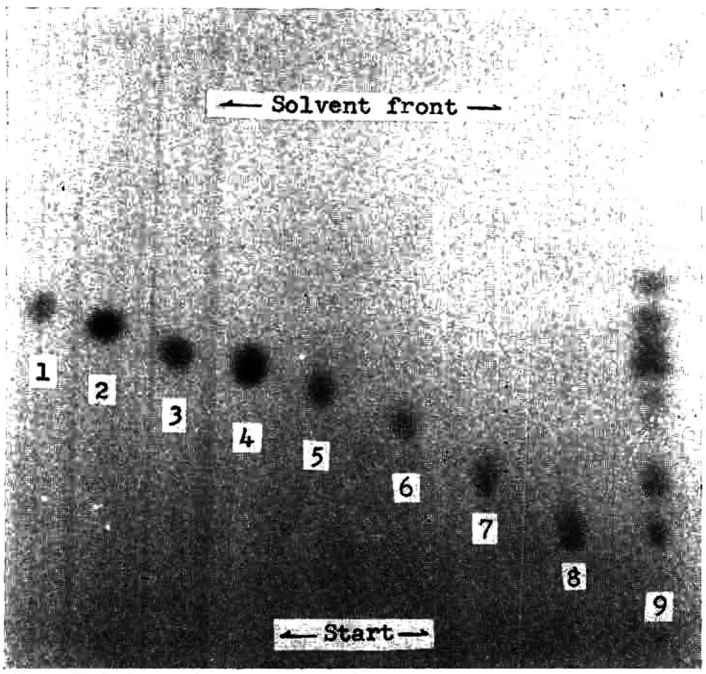

Fig. 1. Thin-layer chromatogram of carbohydrates. The chromatogram was developed in solvent system propanol–water (85:15). The spots were detected with the *o*-aminodiphenyl–orthophosphoric acid reagent. Carbohydrates: (1) rhamnose, (2) xylose, (3) mannose, (4) glucose, (5) galactose, (6) maltose, (7) lactose, (8) raffinose and (9) mixture of (1)–(8).

bria are involved between the adsorbent layer and developing solvent as the sugar-bisulfite compounds move up on the plates. The differences in the development pattern of carbohydrates on plates and on resin columns may be due to factors affecting the migration.

Concomitantly with the experiments on separation various color reagents were being tested for the thin-layer chromatographic detection of carbohydrates. As seen in Table I, the *o*-aminodiphenyl–orthophosphoric acid and thymol–sulfuric acid reagents are of special interest because of the different colors obtained with aldo- and keto-sugars, some of these sugars having R_F values which do not always permit unequivocal identification. The phenol–sulfuric acid reagent gives wider color differences between various carbohydrates. To test the sensitivity of these reagents, aliquots of 0.1, 1, 5 and 20 μg each of fructose, glucose and xylose were spotted on a plate and sprayed with the reagents. Even at a level of 0.1 μg of the carbohydrates the colors were clear and identifiable. The speed of color development of fructose has been found to be generally faster than that of the aldoses. Another distinct advantage of these spray reagents is the lack of background color. The reagents react with the test carbohydrates and the resultant color stands out clearly against a white background. The characteristic colors obtained with keto-sugars by dimedone–orthophosphoric acid reagent are also noteworthy. The reagent reacts only with the test keto-sugars and not with the aldo-sugars, the one exception being 2-deoxy-D-glucose. The minimum sensitivity of the reagent was also 0.1 μg of fructose. On the other hand, with the

carbazole–sulfuric acid reagent a violet color on a blue background was observed for all of the test carbohydrates. The reaction was less sensitive than with the other reagents.

The reactions of carbazole[15], phenol[16] and thymol[17] with carbohydrates in the presence of strong sulfuric acid have been modified for use as methods for the estimation of carbohydrates and these reactions give characteristic colors which have a different absorption maximum for pentose and hexose. It is therefore of interest to note the similarities and differences between pairs of carbohydrates when these reagents were applied to colorimetric determination and detection in thin-layer chromatography. TIMELL *et al.*[18] have demonstrated that *o*-aminodiphenyl in glacial acetic acid gives a characteristic color reaction with aldo-sugars but not with fructose and sucrose in comparable concentration. The results for the color reaction of the dimedone–orthophosphoric acid reagent with carbohydrates parallel the experience of ADACHI using paper chromatography[10].

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SUMMARY

A rapid method for separating carbohydrates by means of thin-layer chromatography on silica gel G mixed with a small amount of sodium bisulfite has been developed. The solvent system propanol–water (85:15) gave the best resolution of the carbohydrates but did not separate some pentoses. Five spray reagents, *viz.* *o*-aminodiphenyl–orthophosphoric acid, carbazole–sulfuric acid, dimedone–orthophosphoric acid, phenol–sulfuric acid and thymol–sulfuric acid, were used for the detection of carbohydrates. Quantities as small as 0.1 μg could be easily detected with these reagents, with one exception, *viz.* carbazole–sulfuric acid reagent. The carbohydrates gave characteristic colors with the reagents, thus making identification easier.

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THE SEPARATION OF SIMPLE SUGARS BY CELLULOSE THIN-LAYER CHROMATOGRAPHY*

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INTRODUCTION

Thin-layer chromatography (TLC) is a useful tool for the rapid separation of compounds of biological interest. Although many different sorbents are available which are suitable to TLC, the majority of the workers have used silica gel with a binder for the sorbent layer[1]. One group of compounds for which silica gel has not been a satisfactory medium is the naturally occurring free sugars. Poor separation of some of the more common sugars and the low capacity of the plates are two disadvantages in this application.

Although the simple sugars have been separated for many years using paper chromatography, a major disadvantage is the long elution time required with the associated problems of temperature fluctuations[2]. When cellulose thin layers are used, the advantages of the partitioning properties of cellulose and the increased loading of sample are coupled with the separations which are characteristic of thin-layer chromatography. RANDERATH[3] has found cellulose thin-layer chromatography superior to paper chromatography in the separation of nucleotides. The authors have found no more than one single paper[4] in the literature which deals with the separation of a few simple sugars using cellulose layers for thin-layer chromatography.

In terms of solute detectability and time, it is advantageous to find a solvent or solvents which will give good separation of sugars in one dimension. Since cellulose thin-layer plates possess the same partition properties as paper, it seemed advisable to evaluate several solvents previously used for the separation of sugars by paper chromatography to determine their applicability for the separation of sugars on cellulose layers.

Nine solvents were evaluated in this study. Special emphasis was placed upon the property of the solvent to separate sucrose, glucose, and fructose in one dimension without a long period of pre-saturation of the plate. These sugars are of principal interest because they are the most commonly occurring free sugars in higher plants.

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* Technical Paper No. 889 of the Agricultural Experiment Station, University of Arizona, Tucson. The mention of specific sources of equipment or products to the exclusion of others that may be suitable should not be considered as an endorsement by the University of Arizona of a particular manufacturer or product.

EXPERIMENTAL

(A) *Solvents*

The following solvents were evaluated:

(1) Formic acid–methyl ethyl ketone–*tert.*-butanol–water (15:30:40:15, v/v)[5].
(2) Ethyl acetate–pyridine–water (2:1:2, v/v)[6].
(3) Ethyl acetate–isopropanol–water (65:23.5:11.5, v/v)[7].
(4) *n*-Butanol–acetic acid–water (6:3:1, v/v)[7].
(5) Methyl ethyl ketone–acetic acid–methanol (3:1:1, v/v)[7].
(6) Ethyl acetate–acetic acid–water (3:2:3, v/v)[8].
(7) *n*-Butanol–pyridine–water (45:25:40, v/v)[8].
(8) *iso*-Propanol–pyridine–acetic acid–water (8:8:1:4, v/v)[9].
(9) Phenol aq. (ca. 90 %)–water (10:1.25, v/v) + 0.002 % 8-hydroxyquinoline[10].

(B) *Detection reagent*

The detection reagent used was 2-aminodiphenyl–oxalic acid dissolved in 85 % ethanol[9]. This reagent locates disaccharides as well as hexoses and pentoses. The 2-aminodiphenyl is no longer commercially available because of its suspected carcinogenic properties. Several methods of preparation are given in the literature[11,12].

(C) *Preparation of plates*

The plates were washed with a detergent, rinsed well with tap water, followed by distilled water and finally methanol (analytical grade).

The cellulose slurry was prepared as follows: 15 g of cellulose 300 MN (Macherey, Nagel & Company) were mixed with 90 ml of a deionized water–methanol solution (5:1, v/v) by adding small portions of the solution to the powder and stirring well. A homogeneous slurry resulted. This was sufficient to cover five 20 × 20 cm plates and two 5 × 20 cm plates. A 0.37 mm thick layer was applied with an adjustable applicator (Desaga).

The plates were always dried in a hood for 2 h and then stored in a desiccator cabinet overnight before use. (The relative humidity in the laboratory seldom was over 15 %.)

(D) *Sample preparation*

The sugar solutions were made by dissolving 1 mg of sugar in 10 ml of 10 % isopropanol. A mixture of glucose, fructose and sucrose was prepared in the same way. The following sugars were studied:

Disaccharides: sucrose, lactose, cellobiose, maltose;
Aldohexoses: β-D-glucose, D-mannose, D-galactose;
Ketohexoses: D-fructose, L-sorbose;
Aldopentoses: D-arabinose, L-arabinose, D-lyxose, L-xylose, D-ribose.

(E) *Chromatographic procedure*

The samples were spotted at 1 cm intervals using a micropipette. The pentoses were applied at 30 μg per spot and the others at 40 μg per spot. The origin was 2 cm above the bottom edge of the plate. The film was broken 15 cm above the origin. The film was also broken vertically 0.5 cm from each side to eliminate edge effects[13].

Solvent was placed in the tank 15 min before the plates were added. The laboratory temperature was 23°.

After the plates were developed and dried they were sprayed with the detecting reagent. The sugars were located by heating the plates with an industrial hot air drier. A lighter background resulted by this procedure than when heated for 10 min in an oven at 110°.

RESULTS AND DISCUSSION

These nine solvents ascend more slowly on cellulose layers than on silica gel layers. The development time is still rapid when compared to the usual developing times of 24 h or longer for paper chromatography. A slightly faster development time can be achieved using cellulose with $CaSO_4$ as a binder. No other differences were observed in this laboratory between cellulose with and without a binder.

Table I lists the characteristic colors produced by the spray reagent. The order of appearance and relative intensities are also given. These results were the same for all the solvents tested.

Table II lists the $R_F \times 100$ values for the various solvents. The $R_G \times 100$ values for solvent 1 also are given. In this case, the plate was developed twice in the same direction. Several of the solvents gave severe streaking, especially the more volatile, faster running solvents.

Formic acid–methyl ethyl ketone–*tert.*-butanol–water is the preferred solvent. The spots containing pentoses enlarged to about twice the diameter of the original spot after being developed twice. The higher molecular weight sugars diffused even less. All spots were nearly circular with no bearding or tailing whereas for all of the other solvents tested the spots were elongated and bearded, and there was an occasional double spot. Fig. 1 shows the results of a 15 cm development using solvent 3.

Since the R_F values are low in the formic acid–methyl ethyl ketone–*tert.*-butanol–water solvent, multiple development can be used to advantage[14]. Fig. 2 shows the amount of separation after one 15 cm development in solvent 1.

Fig. 3 shows the increased separation achieved after two developments in the same direction.

The literature to date indicates that the use of thin-layer chromatography for the separation of sugars is inferior to paper chromatography because of the small quantities of sugars that can be used. These results were obtained on silica gel. With the solvent 1–cellulose substrate combination, mixtures containing 100 μg or more of sucrose, glucose, and fructose can be separated using the multiple development technique. The upper limits for the separation of most pentoses appear to be 50 μg.

Galactose can be separated from glucose using this system. After two developments with solvent 1, about 15 μg of galactose can be separated from the same quantity of glucose. If larger amounts of these sugars are present, three or more developments may be required. With the phenol–water system galactose moves farther than glucose, permitting a satisfactory separation of as much as 50 μg of each sugar in one development.

Using solvent 1, the sugars are separated into classes, *i.e.*, trisaccharides remain nearest the origin, disaccharides above them, aldohexoses next, etc.

TABLE I

CHARACTERISTIC COLORS OF SUGARS SPRAYED WITH 2-AMINODIPHENYL

Class	*Color*	*Order of appearance*	*Relative intensity*
Disaccharides	Light tan	3	3
Aldohexoses	Very dark brown	2	2
Ketohexoses	Green, change to green-brown on prolonged heating	4	4
Aldopentoses	Red	1	1

TABLE II

R_F VALUES OF SUGARS IN THE NINE SOLVENTS

	$R_F \times 100$									$R_G \times 100$
Compound/solvent	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*1*
Sucrose	10	20	4	S	0	63	40	67	37	65
Lactose	4	17	1	5	0	56	31	39	37	26
Maltose	6	S	2	7	0	62	34	48	34	38
Cellobiose	5	27	1	4	0	60	33	45	32	32
β-D-Glucose	19	S	S	17	0	63	40	47	35	100
D-Mannose	23	40	S	S	0	65	43	60	40	123
D-Galactose	17	S	S	S	0	60	37	53	40	91
D-Fructose	24	S	11	22	0	62	41	61	47	130
L-Sorbose	23	S	10	20	0	63	41	60	37	123
D-Arabinose	27	40	S	23	0	63	41	61	50	145
D-Lyxose	33	48	S	S	0	67	46	57	46	170
L-Xylose	30	47	S	25	0	67	46	65	41	160
D-Ribose	39	53	S	S	0	69	49	69	57	191
L-Arabinose	29	39	S	23	0	63	40	60	51	151
Mixture: α-D-Glucose–D-Fructose–Sucrose	Y	N	N	N	N	N	N	N	Y	Y
Approximate time (h) for solvent to travel 15 cm at 23°	3	2	3	4	½	3	4	4	6	6 total

N = No separation of the mixture; Y = Yes, mixture separated; S = Badly streaked.

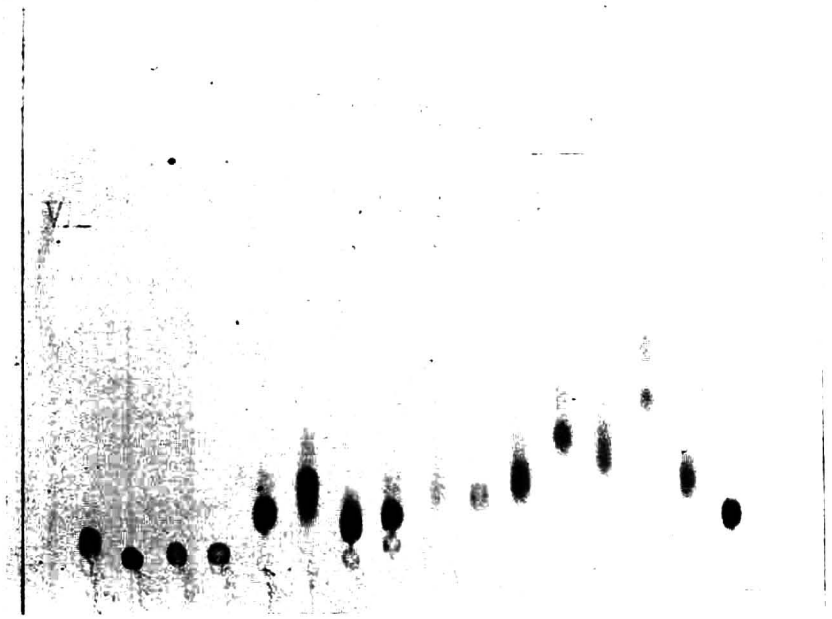

Fig. 1. A 15 cm development in ethyl acetate isopropanol–water showing typical bearding and poor separation. From left to right the sugars are: sucrose, lactose, maltose, cellobiose, D-glucose, D-mannose, D-galactose, mixture, D-fructose, L-sorbose, D-arabinose, D-lyxose, L-xylose, D-ribose, and L-arabinose.

Fig. 2. A single 15 cm development in the formic acid–methyl ethyl ketone–*tert.*-butanol–water solvent. The sugars are in the same sequence as in Fig. 1.

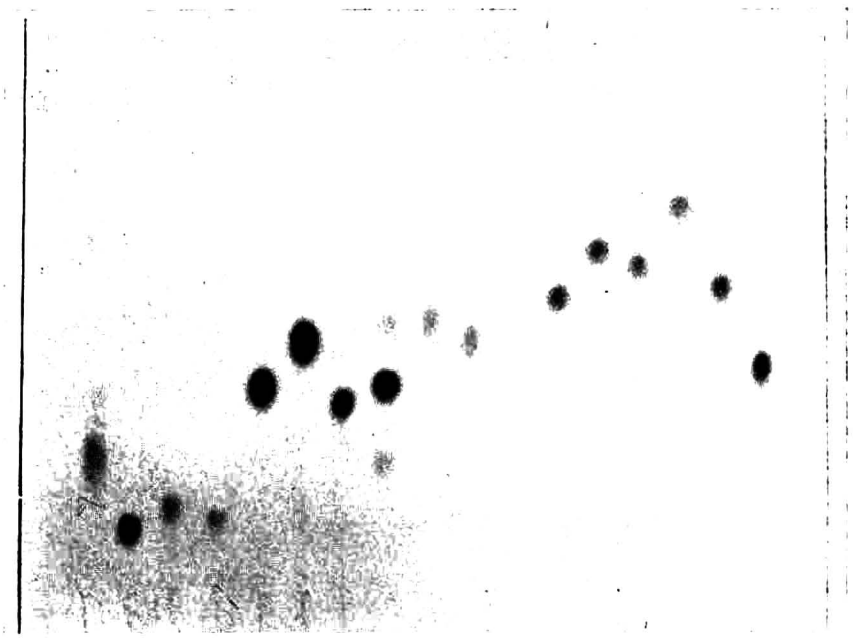

Fig. 3. The separation of sugars achieved after two developments of 15 cm. The sugars are in the same sequence as in Fig. 1.

ACKNOWLEDGEMENTS

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SUMMARY

Several solvents previously used in paper chromatography for the separation of sugars were evaluated for use with cellulose thin-layer plates. Several simple sugars which were difficult to separate by one-dimensional chromatography on either paper or silica gel can be separated on cellulose using a solvent of formic acid–methyl ethyl ketone–*tert.*-butanol–water.

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THIN-LAYER SOLUBILIZATION CHROMATOGRAPHY

I. PHENOLS

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INTRODUCTION

Solubilization chromatography is a method for separating organic nonelectrolytes having a small solubility in water. It is a type of partition chromatography which employs ion-exchange resin as the stationary phase and aqueous solutions of organic compounds as the mobile phase. With this technique, a series of phenols[1] (among other compounds[2,3]) have been separated by elution with acetic acid through a column of strongly-acidic cation-exchange resin. Mixtures of phenols were later separated by development with acetic acid on a sheet of filter paper loaded with the same type of resin (paper solubilization chromatography[4]). This report demonstrates the combination of solubilization chromatography and thin-layer chromatography and its application to phenol separations.

Phenols of various kinds have been chromatographed on thin-layer plates made from a wide range of sorbents, for example silica gel[5], polyamides[6], and unbound aluminum oxide[7]. Thin layers of ion-exchange resin have apparently not been employed. In fact, only one report of the use of ion-exchange resins in thin-layer chromatography has come to our attention. BERGER *et al.*[8] separated three radioactive halides by development with molar sodium nitrate and three organic dyes by development with a 4:4:1 mixture of acetic acid, methanol and acetone. To prepare their layers, however, they used equal parts of cellulose powder containing 5% plaster and 200–400 mesh anion- or cation-exchange resin. In the present work, our layers are composed only of resin and binder.

EXPERIMENTAL

Apparatus and reagents

"Chromatofilm" thin-layer chromatography apparatus, manufactured and supplied by Research Specialties Co., Richmond, Calif., was used throughout this work.

The ion-exchange resins employed were the strongly-acidic cation-exchange resin, Dowex 50W-X8, 200–400 mesh (Lot No. 4673-30; Dow Chemical Co., Midland, Mich.), and the strongly-basic anion-exchange resin, Dowex 1-X4, 200–400 mesh (Lot No. 4697-27). The Dowex 50 was used as supplied except for some experiments in which the ammonium form of the resin was employed. Conversion from the hy-

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drogen form was accomplished by passing a saturated solution of ammonium chloride through a column of the resin until the pH of the effluent and influent were identical. The resin was then washed with distilled water. The Dowex 1 was cleaned and converted to the chloride form by successively passing 150 ml of 1.5 *M* sodium hydroxide, 150 ml of distilled water, 150 ml of 1.5 *M* hydrochloric acid, and a final 150 ml of distilled water through a column of resin.

All chemicals used were of the best grade commercially available. Developing solutions were prepared by proper dilution of reagent-grade acetic acid and methanol. Individual test solutions of the phenols were prepared by dissolving 0.20 g of catechol, resorcinol, phenol, *o*-cresol, *m*-cresol or *p*-cresol in distilled water (or the minimum necessary amount of methanol) and diluting to 25 ml with additional water. The first three phenols could be completely dissolved in water, but the cresols could not. A mixture solution of the six phenols was prepared by the combination of individual solutions containing 1.2 g of each phenol in 25 ml of water including a minimum amount of methanol if necessary.

Preparation of layers

A slurry of ion-exchange resin was prepared by a modification of the method used by SMITH AND FOELL[9] for silica gel. One gram of soluble starch and 30.0 ml of distilled water were added to 25.0 g of dry hydrogen-form Dowex 50 resin in a 250-ml erlenmeyer flask. The flask was heated with occasional stirring on a steam bath for 20 min at 75–80°. The flask was allowed to cool and more water, usually less than 10 ml, was added to give a slurry of the proper consistency. The consistency of this mixture is very important: it should be thin enough to allow five plates to be coated but thick enough so that the coating does not run after application to the plates. With the Dowex 50 in the ammonium form and the Dowex 1, the slurry was prepared using wet resin from the column, and less water (10–20 ml) was added before heating with the starch.

Glass plates, 8 in. × 8 in., were soaked overnight in "cleaning solution", rinsed with distilled water, and dried. Five of these plates were positioned on the aligning tray. The variable-thickness spreader was adjusted, using gauges supplied with it, to give a layer of 0.3 mm thickness. It was found that thicker layers tended to crack more readily and that thinner layers were difficult to apply evenly. The slurry was mixed well and poured into the spreader, which was then drawn over the plates at a slow, steady rate. The plates were allowed to stand in place for 15 min and were then stored in a storage rack until used.

In the beginning, attempts were made to dry the plates in an oven after the standing period (at 60–70°, or by gradually increasing the temperature from 25° to 70°), but this usually resulted in cracked layers. Calcium sulfate was tried in place of starch as the binder, but this also usually resulted in cracked layers being formed. The use of calcium sulfate is not desirable anyway because of the possibility of ion exchange occurring.

After some practice and experience, it was possible to prepare good layers of resin almost every try by use of the procedure described above.

Spotting techniques

Initial zones of the test solutions were applied to the plates using Peerless

wood applicator sticks (Diamond Match Co., New York 17, N.Y.). Compared with micropipettes, it was easier in this way to apply a detectable amount of solute to a smaller area without disturbing the layer. Three dabs of test solution were used for each spot; this corresponded to about 5 μl. The plates were consistently developed in the direction opposite to which the spreader was moved in the preparation of the layers. The solutes were spotted on an origin line 18.0 cm from the top of the plate, and the solvent was allowed to rise to within 3.0 cm of the top. The distance of development was therefore 15.0 cm in all cases. A line was drawn across the layer with a grease pencil to mark this position 3.0 cm from the top. No line was drawn at the origin, but the plastic template which had lateral markings was used as a guide in placing the initial solute spots. Up to seven initial zones, one inch apart, were placed on one plate. The spots were air dried for 15 min after application.

Whenever mixtures were chromatographed, individual reference zones of each constituent in the mixture were placed on the same plate. Mixtures which were studied included the one containing all six phenols described above, as well as all possible binary mixtures of the six phenols. These two-component mixtures were prepared on the plate by successive application of individual sample solutions to the same area of the plate, with drying in between.

Development

The developing chamber (Model-200D) was lined with Whatman No. 1 filter paper. Three hundred ml of developing solvent were poured down the sides of the chamber to completely saturate the paper. The chamber was covered for 20 min to allow equilibration of the atmosphere inside. One or two plates were placed on the A-frame in a near-vertical position with the origins at the bottom. The plates were put into the chamber as quickly as possible so that it was uncovered for a minimum amount of time. The solvent was allowed to rise until it reached the line drawn across the top of the plates. For irregular solvent fronts, the solvent was permitted to rise until the average of the front was at the line. The plates were then removed and air-dried in a horizontal position for 15–20 min, depending on the solvent.

In some cases, unidirectional multiple development was employed. After the first development, the plate was removed, air-dried, and redeveloped. Up to three consecutive developments were tried.

Detection

The dried plates were sprayed with a diazotized benzidine solution. This was prepared by mixing 20.0 ml of a stock benzidine solution (1 l of solution containing 5.0 g benzidine and 14.0 ml conc. HCl) with 20.0 ml fresh 10 % sodium nitrite solution at 0° with constant stirring. This reagent was stable for only 2–3 h. Some of the spots appeared at once, others after several hours. Consequently, the plates were allowed to stand overnight before evaluation.

During preliminary work other reagents were tried, but none proved as successful for detecting all of the phenols as the one above. These included diazotized sulfanilic acid, sodium molybdate, acidic potassium permanganate, ferric chloride plus potassium ferricyanide, and *p*-nitroaniline followed by sodium carbonate. The usual vigorous, corrosive sprays suggested for thin-layer chromatography attacked the organic layer and were useless.

RESULTS AND DISCUSSION

R_F values were computed by dividing the distance moved by each phenol (measured to the front edge of the zone) by the distance moved by the solvent front. For multiple chromatography, the R_F values were determined from the total distances the solute and solvent moved in the several developments.

Solubilization chromatography on cation-exchange resin layers

(1) *Hydrogen-form resin.* In the hope of being able to compare the results obtained with those from earlier work in columns[1] and on paper[4], the first chromatographic system tried included Dowex 50 in the hydrogen form as the stationary phase and aqueous solutions of acetic acid as the mobile phase. The acetic acid caused severe cracking of the layers and very irregular solvent fronts. Therefore, we switched to aqueous solutions of methanol (which had been used in earlier column studies of ketones[2]) and found the resultant chromatograms were much easier to spray and evaluate.

Table I shows the R_F values obtained with methanol. Times for development ranged from 1.0–1.5 h. At lower methanol concentrations the spots were generally less than 2.0 cm in length. At higher methanol concentrations, catechol and resorcinol

TABLE I

R_F VALUES ON DOWEX 50W-X8 (H^+) LAYERS

Phenol	*R_F values*									
	Concentration of methanol (M)									
	0.0		*1.0*		*2.0*	*3.0*	*4.0*	*5.0*	*6.0*	*7.0*	
Catechol (orange)	0.41	0.41	0.47	0.45	0.51	0.58	0.60	0.59	0.46	0.66
Resorcinol (yellow)	0.43	0.43	0.47	0.46	0.58	0.58	0.59	0.66	0.66	0.71
Phenol (purple)	0.27	0.29	0.30	0.30	0.31	0.32	0.30	0.39	0.37	0.41
o-Cresol (light blue)	0.15	0.23	0.25	0.24	0.22	0.23	0.26	0.28	0.39	0.39
p-Cresol (yellow)	0.15	0.20	0.20	0.21	0.21	0.21	0.24	0.29	0.26	0.35
m-Cresol (purple)	0.19	0.21	0.23	0.23	0.22	0.24	0.31	0.32	0.33	0.30

gave zones up to 3.0 cm in length. The colors of the zones after spraying with diazotized benzidine are shown in parentheses. Duplicate runs are shown for 1.0 and 7.0 *M* methanol to indicate the degree of reproducibility to be expected. In general, reproducibility was best for lower concentrations of methanol.

The results shown in Table I for starch-bound thin layers of cation-exchange resin are generally comparable to those obtained on columns of resin and resin-papers. In columns[1], the distribution coefficient (*C* value) for a given phenol decreased as the molarity of eluting solvent increased. This indicated a decrease in affinity for the stationary resin phase. On cation-exchange paper[4], R_F values for a given phenol increased with an increase in concentration of the developing solvent, again indicating a decrease in affinity for the resin phase. Table I shows a general increase in R_F value for each phenol as the molarity of methanol increases. This trend was expected

because as the molarity of organic constituent in the mobile phase increases, it can better compete with the resin phase for the solute molecules. The R_F values in Table I rise in a parallel manner for each of the phenols. In column solubilization chromatography[1], plots of log C *vs.* molarity of eluent were a series of more or less parallel straight lines, as were plots of R_F *vs.* molarity of developer in paper solubilization chromatography[4]. As a consequence, a separation of a mixture of phenols which was possible with any one concentration of methanol could not be enhanced by changing solvents, as was true for the column solubilization chromatography of most compounds studied[1–3].

Another interesting comparison among the data for phenols on columns of resin, resin-papers and on thin layers of resin is their affinity for the stationary phase when the solvent was water. Focusing on just four phenols, the order of increasing affinity for the resin-paper as measured by decreasing R_F values was *o*-cresol > phenol > resorcinol > *p*-cresol[4]. On thin layers of resin, the R_F sequence was resorcinol > phenol > *o*-cresol = *p*-cresol. Only two of these were studied on columns of resin[1], and *o*-cresol had much more affinity than phenol. Considering the many different forces which can contribute to the retention of a phenol by the various stationary phases, there is no reason to believe that the migration sequences should be the same. Much more work is needed to clarify the results presented above, but they seem to indicate that interactions between the phenols and the cellulose and/or binder of the resin-paper may be much more important than any interactions with the starch, and that starch-bound layers of resin may function in a manner analogous to a column of resin alone.

With the results of Table I, separations of binary mixtures of the phenols were planned and performed as follows: by development with water, phenol and *p*-cresol were separated by 0.80 cm and phenol and *m*-cresol were separated by 0.30 cm (distances given are between the leading edge of the slower-moving zone and the trailing edge of the faster-moving zone; all separations were performed at least three times and the distances given are the average for all the replicates; in every run, separation was achieved every time); by development with 3.0 M methanol, phenol and *o*-cresol were separated by 0.50 cm and *m*-cresol and resorcinol were separated by 1.7 cm; by development with 4.0 M methanol, catechol and *o*-cresol were separated by 1.3 cm, catechol and *p*-cresol were separated by 1.6 cm and catechol and *m*-cresol were separated by 1.2 cm; by development with 5.0 M methanol, catechol and phenol were separated by 1.8 cm; by development with 6.0 M methanol, *o*-cresol and resorcinol were separated by 3.2 cm and *p*-cresol and resorcinol were separated by 3.0 cm; and by development with 7.0 M methanol, phenol and resorcinol were separated by 2.3 cm. Besides these, the six-component test solution was subjected to double development with 4.0 M methanol. Three zones resulted: a blue-purple zone containing the cresols (R_F 0.21) was separated by 1.2 cm from the purple phenol zone (R_F 0.29) which was separated by 1.0 cm from an orange zone (R_F 0.39) containing resorcinol plus catechol.

Attempts to separate the three cresols from each other by single development with concentrations of methanol greater than 7.0 M or by multiple development at various solvent concentrations all failed. Likewise, conditions within the present system could not be found by which catechol could be consistently separated from resorcinol.

(2) *Ammonium-form resin.* Table II shows the results of developing the phenols with methanol on Dowex 50 in the ammonium form. Development times ranged from 55 to 70 min. Spot sizes varied from less than 1.0 cm to almost 4.0 cm in length,

TABLE II

R_F VALUES ON DOWEX 50W-X8 (NH_4^+) LAYERS

Phenol	*R_F values*				
	Concentration of methanol (M)				
	0.0	*2.0*	*4.0*	*6.0*	*8.0*
Catechol (brown)	0.24	0.24	0.29	0.32	0.34
Resorcinol (red)	0.25	0.27	0.29	0.43	0.43
Phenol (yellow)	0.21	0.20	0.25	0.32	0.34
o-Cresol (yellow)	0.15	0.15	0.16	0.21	0.29
p-Cresol (yellow)	0.13	0.14	0.19	0.22	0.27
m-Cresol (yellow)	0.15	0.15	0.19	0.27	0.27

resorcinol and catechol being the most diffuse. Note that the colors of the developed zones after spraying with diazotized benzidine were different on the different resin form.

Similar to the results on the hydrogen-form resin, the R_F values increased with increasing molarity of methanol in a roughly parallel manner for each phenol. Again, the solubilizing effect of the methanol is readily apparent. The order of affinity of the phenols for the ammonium-form resin was the same as for the hydrogen-form (resorcinol < catechol < phenol < cresols), but the actual R_F values were substantially lower. This greater affinity for the ammonium-form layer compared with the hydrogen-form layer is just opposite to the results obtained in column studies of the effect of the counter ion of Dowex 50 when various ketones were eluted with water[10]. No explanation can be offered for these differences, unless the starch is involved.

This system exhibited much less selectivity toward the phenols. No separations could be performed with any molarity of methanol as the developer.

Solubilization chromatography on anion-exchange resin layers

Table III shows the results of developing the phenols with methanol on layers of Dowex 1-X4 in the chloride form. Times for development ranged from 25 to 75 min, being much faster with high solvent concentrations. The developed zones were generally less than 2.0 cm in length, becoming only slightly more diffuse with increasing concentration of methanol. R_F values for all concentrations of methanol between 0.0 and 6.0 *M* were 0.10 or less.

The much greater affinity of the phenols for the resin phase as evidenced by the very low R_F values throughout Table III was expected. Dowex 1 is more organic in nature (more carbon atoms per functional group) than Dowex 50, and one of the most important interactions[11] between the phenols and the stationary phase was undoubtedly the London dispersion forces involving the hydrocarbon parts of the resin.

It is interesting that the order of the R_F values on the anion-exchange resin tends to reverse trends established on the cation-exchange resin. At high concen-

TABLE III

R_F VALUES ON DOWEX 1-X4 (Cl^-) LAYERS

Phenol	*R_F values*					
	Concentration of methanol (M)					
	0.0	*6.0*	*7.0*	*12*	*19*	*22*
Catechol (yellow)	0.09	0.10	0.10	0.17	0.27	0.31
Resorcinol (red-brown)	0.07	0.08	0.08	0.13	0.22	0.25
Phenol (orange)	0.09	0.11	0.12	0.21	0.37	0.37
o-Cresol (orange)	0.05	0.08	0.09	0.16	0.37	0.45
p-Cresol (orange)	0.06	0.08	0.08	0.17	0.39	0.45
m-Cresol (orange)	0.07	0.08	0.09	0.17	0.38	0.40

trations of methanol, the cresols exhibited the least affinity for the anion exchanger, having R_F values about 1.5 times as large as resorcinol and catechol, which always had the highest R_F values on the cation exchanger.

Only with very high concentrations of methanol did the phenols migrate significantly. The system was selective enough, however, to allow various separations to be performed. For example, with 19 *M* methanol (75 %), resorcinol was separated from every phenol except catechol by at least 0.70 cm, and catechol was separated by 0.70 cm from *p*-cresol. Triple development with 22 *M* methanol (90 %) separated catechol (R_F 0.12) by at least 0.50 cm from every phenol except resorcinol. Even catechol and resorcinol, which showed little differential migration in the other systems, were substantially separated. Further development was impossible because the layers cracked after the third solvent pass.

Salting-out chromatography on cation-exchange resin layers

Salting-out chromatography[12] is a method by which water-soluble nonelectrolytes have been separated by elution through ion-exchange resins with aqueous salt solutions as eluents. We decided to develop the phenols by thin-layer salting-out chromatography in a system including Dowex 50 in the ammonium form as the stationary phase and aqueous solutions of ammonium sulfate as the mobile phase.

TABLE IV

R_F VALUES ON DOWEX 50W-X8 (NH_4^+) LAYERS

Phenol	*R_F values*		
	Concentration of ammonium sulfate (M)		
	0.0	*1.0*	*3.8*
Catechol	0.24	0.13	0.070
Resorcinol	0.25	0.15	0.090
Phenol	0.21	0.11	0.050
o-Cresol	0.15	0.070	0.030
p-Cresol	0.13	0.060	0.020
m-Cresol	0.15	0.070	0.030

The results for the individual phenols are shown in Table IV. Times for development ranged from 50 to 105 min. The developed zones were 0.60 to 2.5 cm in length, being more compact as the salt concentration increased. The colors of the sprayed zones were the same as for the ammonium-form layers developed with methanol. At high salt concentrations, the solvent front was unusually irregular.

These preliminary experiments indicate that salting-out chromatography on thin layers of ion-exchange resin is a feasible procedure worthy of future detailed study. We did not expect to be able to separate the phenols with it because their R_F values when developed with water (R_{F0}) were too low. The R_F values did decrease as expected with increasing salt concentration but apparently not selectively. It should be possible to find selective systems for producing separations of nonvolatile water-soluble solutes with high R_{F0} values. The salt eluents would tend to decrease and separate the R_F values if the results were comparable to column salting-out chromatography.

COMMENTS

The purpose of this research was to present a new separation procedure combining thin-layer chromatography with partition chromatography on ion-exchange resins, and to compare the results of this procedure with results from similar types of chromatography reported earlier. A limited number of phenols were chosen for study, but the method would undoubtedly be applicable to virtually any uncombined phenols or derivatives which could be detected successfully. In fact, additional phenols were examined, although not in detail, and showed evidence of useful migration in several of the systems. These included pyrogallic acid, phloroglucinol, *o*-nitrophenol, *o*-phenylphenol and *p*-hydroxybenzaldehyde. Further work is now in progress to extend this separation technique to other classes of compounds and to study other experimental variables in the process.

As in all differential migration methods, the behavior of individual solutes is subject to considerable variation, such as in R_F and the size of individual zones. This is especially true in thin-layer chromatography because the preparation of nearly identical layers from day to day is a genuine art. The reproducibility of R_F values shown in Table I (which was typical but not the best we could have shown) is quite good except for two or three values. It was generally possible to reproduce values to ± 0.03 R_F units throughout this work.

The phenols were undoubtedly very slightly ionized during their migrations. It is probable, however, that the actual ion exchange between the stationary phase and these ions was small enough to be neglected compared with other solute–resin interactions, and that the procedure was essentially a separation of nonelectrolytes by a partition process.

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SUMMARY

The R_F values of a series of six phenols have been determined using thin-layer chromatographic techniques on layers of ion-exchange resin. These data have been used to predict and perform the separation of various mixtures of these phenols by development with aqueous solutions of methanol. The procedure is called thin-layer solubilization chromatography, and it is compared to solubilization chromatography in columns and on paper.

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THIN-LAYER CHROMATOGRAPHY OF SOME ^{131}I AND ^{125}I LABELLED IODOPYRIMIDINES AND RELATED NUCLEOSIDES

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INTRODUCTION

Iodinated pyrimidines and nucleosides labelled with radioiodine (^{131}I or ^{125}I) at a very high specific activity are of great interest in studies directed to metabolic chemotherapy of cancer, which are being actively pursued in many laboratories[1]. These investigations are concerned with compounds such as IUDR (5-iodo-deoxyuridine) which interfere specifically with the incorporation of thymidylic acid into DNA, or are able, to some extent, to substitute for thymidylic acid in the biosynthesis of DNA[2]. Other attempts to obtain antimetabolites[3] included studies of the 5-iodo-derivatives of uracil and uridine, and of cytosine and cytidine[4] labelled with radioiodine.

The preparation of labelled iodopyrimidines and nucleosides involves an isotopic exchange reaction in aqueous solution between the inactive iodinated molecules and the radioiodine as iodide ion[5]. A side reaction involving partial hydrolysis of nucleosides often takes place, which gives rise to corresponding pyrimidines. "Free" iodine, as I′, or I_2 is always present in the solutions of radioiodinated pyrimidines or nucleosides, both during the labelling process, because of an incomplete exchange reaction, and in the final products, because of the autoradiolysis. The routine production of labelled pyrimidines and nucleosides requires a rapid and selective method of analysis in order to control the labelling procedure, to measure the exchange yields and to check the purity and identity of the final products.

With this purpose in mind, the separation by thin-layer chromatography, of 5-iodouracil, 5-iodocytosine, related nucleosides and I′ was studied.

A large number of solvent systems were tested on silica gel G and cellulose G, using ascending chromatography, in order to develop a chromatographic method which yields a good resolution of the different components. Since the evaluation of the amounts of the labelled components in a sample is based on the measurement of the areas of the chromatographic peaks obtained by an automatic scanning of the plate and a data recording system, a sharp resolution of the chromatographic spots is an essential condition.

EXPERIMENTAL

Preparation of iodinated pyrimidines and nucleosides

5-Iodouracil and 5-iodocytosine were prepared, according to the method proposed by JOHNSON AND JOHNS[6], by direct iodination of uracil or cytosine in al-

kaline solution at 50–60°. The purity of the products was checked by U.V. spectrophotometry and paper chromatography.

5-Iododeoxyuridine and 5-iodouridine were prepared according to the method proposed by PRUSOFF[7]. A mixture of deoxyuridine (supplied from BDH), iodine, chloroform and nitric acid was refluxed for 2 h until needle crystals of IUDR appeared. The product was recrystallized from hot water and the purity was checked by U.V. spectrophotometry and chromatography. The same procedure was adopted for 5-iodouridine, but the refluxing was continued for 18 h. 5-Iodocytidine was prepared by a modification of the method described by CHANG[8] for the preparation of 5-iododeoxycytidine. Cytidine (100 mg), glacial acetic acid (0.3 ml), iodic acid (35 mg), iodine (60 mg), chloroform (0.1 ml) and water (0.1 ml) were gently warmed for 18 h at 40°. The precipitated white crystals of iodocytidine were washed with ether until all the unreacted iodine was extracted, and then dissolved in glacial acetic acid. Undissolved HIO_3 was discarded and iodocytidine was precipitated by ether and separated by centrifugation. A more detailed description of the preparation procedure and the results of the identity controls will be reported elsewhere[9].

The labelling of iodinated pyrimidines and nucleosides with either ^{131}I or ^{125}I was carried out in aqueous solution by means of an exchange reaction with the radioiodine as iodide ion, at 100° for iodouracil and iodocytosine, at 60° for iodouridine and iododeoxyuridine, and at 40° for iodocytidine.

Preparation of the chromatographic plates

Plates of mirror glass (thickness 3 mm, width 50 mm, length 200 mm) were coated with a 0.3 mm layer of silica gel G or cellulose G by means of a Chemetron apparatus.

Silica gel G plates were dried for 30 min at 110° and allowed to cool in a moisture-free chamber. Cellulose G plates were dried for 40 min at 50°. A sample of the solution to be examined (about 50 μl of a 0.1 % aqueous solution of iodinated labelled pyrimidines or nucleosides) corresponding to approximately 50 μC, was applied by means of a capillary pipette to the plate on a line 2.5 cm from the edge of the plate. The development was carried out by the ascending method in closed rectangular tanks saturated for 1 h with the appropriate solvent system and lined with filter paper for a good equilibration. The developed chromatograms were removed from the tanks, dried and then analyzed by an automatic scanning and recording device.

Scanning and recording system

The chromatographic plates were analyzed by a scanning and recording device, which has been described in detail elsewhere[10].

The plate is laid upon an aluminium slide which runs at a constant speed under a radiation detection unit (phototube Dumont 6292, 1.5 in. × 1.5 in. NaI(Tl) crystal) connected through a single channel analyzer with a recording apparatus (EKCO N522 ratemeter and Speedomax G graphic recorder). In each experiment the shape and the position of the chromatographic spots was previously checked by autoradiography of the plates using Ferrania-X film, exposed for periods of up to 16 h. The film was protected from acidic materials on the plates by thin polyethylene foils. The R_F values of the single components were directly measured on the recorded chromatograms.

RESULTS AND DISCUSSION

The R_F values measured on silica gel and cellulose are listed respectively in Tables I and II. Each R_F value was at first measured for the pure separated compound and then checked again using mixtures.

The results in Tables I and II show that, with a suitable choice of the elution mixture and support, it is possible to attain very satisfactory separations.

TABLE I

R_F VALUES ON CELLULOSE G PLATES

	R_F							
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
5-Iodouracil	0.83	0.77	0.80	0.79	0.71	0.85	0.84	0.83
5-Iodouridine	0.86	0.54	0.59	0.58	0.48	0.85	0.61	0.57
5-Iododeoxyuridine	0.90	0.74	0.78	0.75	0.67	0.74	0.81	0.80
5-Iodocytosine	0.47	0.48	0.48	0.52	0.45	0.61	0.57	0.45
5-Iodocytidine	0.66	0.30	0.13	0.14	0.24	0.62	0.32	0.30
Iodide	1.00	0.35	0.37	0.41	0.91	0.92	0.48	0.59
Time of run (in min)	60	240	240	240	60	60	180	180

a = water; b = *n*-butanol saturated with water; c = *n*-butanol saturated with 0.1 *N* H_3BO_3; d = *n*-butanol saturated with 1 *N* H_3BO_3; e = saturated solution H_3BO_3 in water; f = 0.1 *N* HCOOH; g = *n*-butanol saturated with 0.1 *N* HCOOH; h = *n*-butanol saturated with 0.1 *N* NH_4OH.

When the distribution of the radioactive iodine between the different components is to be evaluated by scanning, thin-layer radiochromatography appears to be very useful for the analysis of mixtures such as iodouridine, iodouracil and iodide (elution mixture (c) on cellulose, (e) on silica gel, as shown in Fig. 1) and iodocytidine, iodocytosine and iodide (elution mixtures (c) and (e) on cellulose, (e) on silica gel as

TABLE II

R_F VALUES ON SILICA GEL G PLATES

	R_F							
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
5-Iodouracil	0.59	0.70	0.69	0.74	0.62	0.56	0.68	0.45
5-Iodouridine	0.72	0.47	0.47	0.50	0.52	0.65	0.47	0.45
5-Iododeoxyuridine	0.57	0.65	0.65	0.70	0.60	0.50	0.63	0.42
5-Iodocytosine	0.58	0.49	0.49	0.52	0.58	0.57	0.53	0.40
5-Iodocytidine	0.68	0.27	0.27	0.29	0.73	0.64	0.42	0.42
Iodide	0.91	0.19	0.18	0.19	0.90	0.81	0.24	0.22
Time of run (in min)	120	300	300	300	120	120	240	240

a = water; b = *n*-butanol saturated with water; *c* = *n*-butanol saturated with 0.1 *N* H_3BO_3; d = *n*-butanol saturated with 1 *N* H_3BO_3; e = saturated solution H_3BO_3 in water; f = 0.1 *N* HCOOH; g = *n*-butanol saturated with 0.1 *N* HCOOH; h = *n*-butanol saturated with 0.1 *N* NH_4OH.

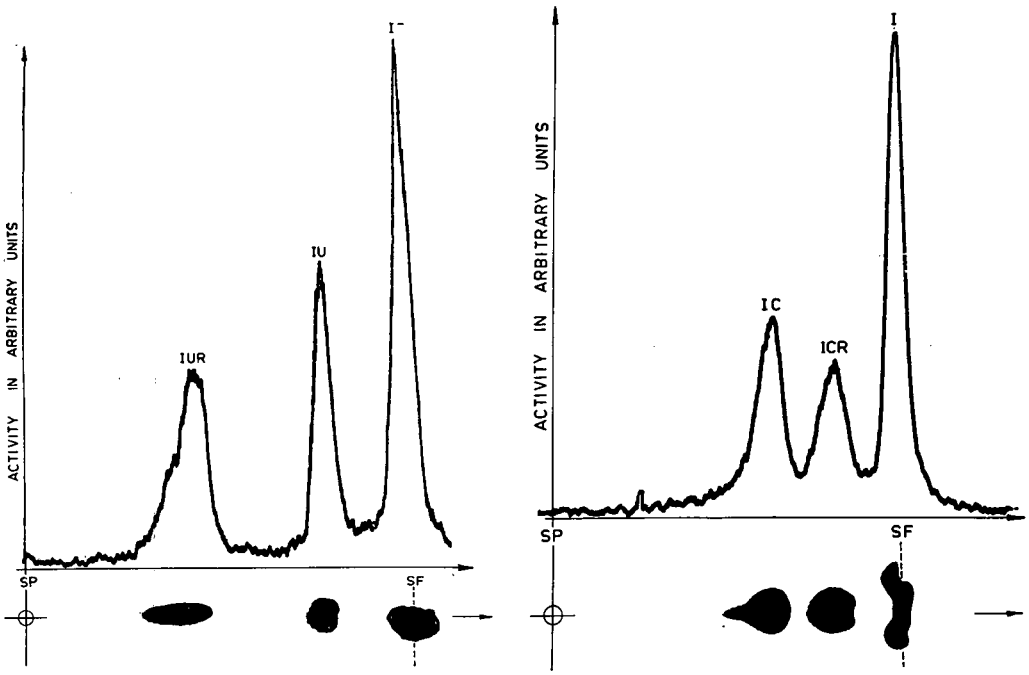

Fig. 1. Scan and autoradiograph of silica gel G plate on which ^{131}I iodouracil (IU), ^{131}I iodouridine (IUR) and iodide ions were separated by eluting with saturated H_3BO_3. Time of run: 60 min.

Fig. 2. Scan and autoradiograph of cellulose G plate on which ^{131}I iodocytosine (IC), ^{131}I iodocytidine (ICR) and iodide ions were separated by eluting with saturated H_3BO_3. Time of run: 120 min.

shown in Fig. 2). Satisfactory separations are obtained for mixtures of the 3 nucleosides and separations of mixtures of labelled ^{131}I iodouridine (IUR), IUDR and ^{131}I iodocytidine (ICR) are shown in Figs. 3 and 4; the iodide ions come from the autoradiolysis of the labelled molecules which were stored for 20 days before analysis. As is shown by autoradiography in Fig. 3, sharp separation can be attained between the spots of ICR, IUR and IUDR; iodide ions interfere with IUR, making the quantitative estimation of this nucleoside more difficult. It must be kept in mind that an improved separation of the recorded peaks could be obtained by lessening the background effect by using a narrower slit with the detection unit.

With a saturated solution of boric acid as solvent, a good resolution of the spots can be attained for IUR, IUDR, ICR and iodide ions, as is shown by the autoradiography reported in Fig. 4.

Since "*in vivo*" degradation of a labelled nucleoside gives rise normally to a pyrimidine and to "free" iodine as I′, this method should be very useful in the study of the biological behaviour of these products, when the analysis of many samples of blood or urine is required. In this case previous desalting of the samples to be analyzed is probably not necessary.

Assays carried out by the authors showed that NaCl in isotonic concentration in the mixture to be analyzed does not induce substantial modifications in the chromatographic separation.

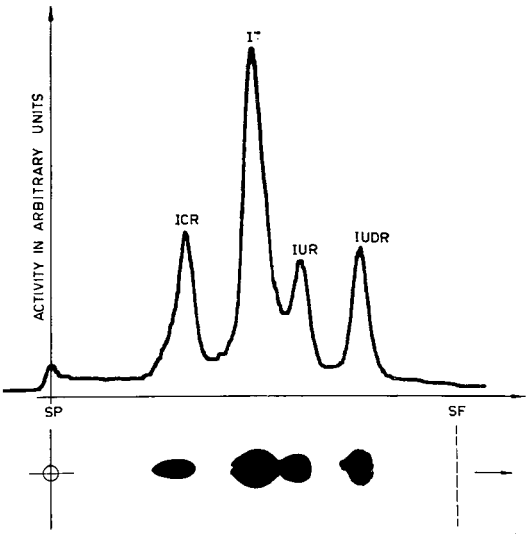

Fig. 3. Scan and autoradiograph of a silica gel G plate on which ^{131}I iodocytidine (ICR), ^{131}I iodouridine (IUR) and ^{131}I iododeoxyuridine (IUDR) were separated by eluting with *n*-butanol, saturated with 1 *N* H_3BO_3. Time of run: 240 min.

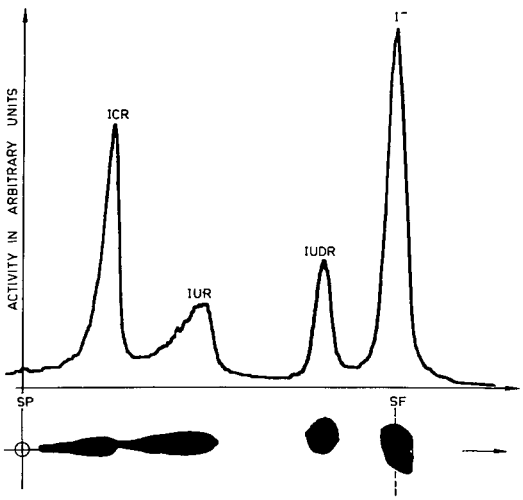

Fig. 4. Scan and autoradiograph of a silica gel G plate on which ^{131}I iodocytidine (ICR), ^{131}I iodouridine (IUR), and ^{131}I iododeoxyuridine (IUDR) were separated by eluting with saturated H_3BO_3. Time of run: 60 min.

SUMMARY

The separation of some radioiodinated pyrimidines and nucleosides of biological interest was studied by thin-layer chromatography.

R_F values of iodouracil, iodocytosine, iodouridine, iododeoxyuridine and iodocytidine on layers of silica gel G and cellulose G were measured for a number of

solvents: *n*-butanol saturated with water, water, *n*-butanol saturated with 0.1 *N* H_3BO_3, *n*-butanol saturated with 1 *N* H_3BO_3, a saturated solution of H_3BO_3 in water, 0.1 *N* HCOOH, *n*-butanol saturated with 0.1 *N* HCOOH and *n*-butanol saturated with 0.1 *N* NH_4OH were used. With a proper choice of elution system and support, a very good resolution of the single spots can be attained and the relative percentages of the labelled components in a sample can be evaluated with a negligible error by radioactive scanning of the chromatographic plate.

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SEPARATION OF CHLOROPHYLLS *a* AND *b* AND RELATED COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

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Chloroplast pigments have been separated by thin-layer chromatography on a mixture of kieselguhr G, silica gel, calcium carbonate, calcium hydroxide and ascorbic acid[1]; on kieselguhr G impregnated with triglycerides[2,3]; and by a lengthy development on silica gel[4]. This report describes a simpler system, using thin layers of cellulose, by means of which mixtures of chlorophylls *a* and *b* and some of their derivatives can be separated. Sucrose and cellulose are frequently employed as adsorbents in column or paper chromatography of chloroplast pigments[5], and the separation of these pigments by thin-layer chromatography on sucrose has been reported[6] since the work on cellulose was started.

EXPERIMENTAL AND RESULTS

Cellulose powder (Macherey Nagel MN 300*, 12 g) and distilled water (68 ml) were mixed with a fast electric stirrer for 4 min. Powders made by Pleuger** (83 M 086, 12 g) and Whatman (CC 41, 25.5 g) were also satisfactory. The slurry was applied with a Desaga spreader* as a 250 μ layer to grease-free glass plates, 20 cm × 20 cm, and allowed to partially dry at room temperature for 25 min. The plates were then placed in a ventilated oven at 105°, horizontally for 10 min and vertically for 25 min. After the plates had cooled, the sample was applied as a spot or streak in dim light and the chromatogram was developed in the dark at room temperature with petroleum spirit (A.R., boiling range 60–80°)–acetone (A.R.)–*n*-propanol (90:10:0.45, v/v/v). This mixture was usually added to the tank, which was lined with chromatography paper, about 20 min before inserting the plate. Development was over a distance of 15 cm and took less than 30 min.

The R_F values for chlorophylls *a* and *b* and some of their derivatives are given in Table I. It must be stressed that these are only approximate values, and that they vary with, for example, the make of cellulose powder and the load, or when the pigments are applied as a mixture rather than as separate spots. The running speeds of the chlorophylls and chlorophyllides, in particular, increase with the load; thus the R_F value for chlorophyll *a* rises to 0.65 for a spot containing 0.6 μg, and to 0.72 for 2.0 μg. The limit of easy detection of the various pigments by daylight was about 0.1 μg, but viewing under ultraviolet light increased the sensitivity for most of the compounds.

* U. K. Distributor: Camlab (Glass), Ltd., Cambridge.
** U. K. Distributor: Townson and Mercer, Ltd., Croydon.

TABLE I

APPROXIMATE R_F VALUES FOR CHLOROPHYLLS AND DERIVATIVES ON THIN LAYERS OF CELLULOSE
The compounds were applied as 0.2 μg spots; the developing solvent, petroleum spirit (60–80°)–acetone–*n*-propanol (90:10:0.45, v/v/v), was allowed to ascend 15 cm beyond the origin.

Compound	R_F	*Compound*	R_F	*Colour of spot (both compounds)*
Pheophytin *a*	0.93	Pheophorbide *a*	0.18	Grey
Pheophytin *b*	0.80	Pheophorbide *b*	0.07	Yellow-brown
Chlorophyll *a*	0.60	Chlorophyllide *a*	0.03	Blue-green
Chlorophyll *b*	0.35	Chlorophyllide *b*	0.02	Yellow-green

In spite of considerable tailing of chlorophylls *a* and *b*, there was a clear zone between these two pigments. Very little tailing occurred with the pheophytins and pheophorbides. Although overloading of the chlorophylls or chlorophyllides resulted in overlapping of some of the pigments, these could be distinguished by their colours (see Table I). Application of the sample as a streak rather than as a spot gave particularly clear results. Small blue-green and yellow-green zones were sometimes observed just ahead of and almost separated from chlorophylls *a* and *b*, respectively, and were presumably the *a'* and *b'* isomers. Protochlorophyll, obtained from the inner coats of marrow seeds, ran between chlorophylls *a* and *b* but tailed badly into *b*. The separation of carotenoids was not studied in detail, but with an extract of bean leaves β-carotene ran between pheophytin *a* and the solvent front, and major xanthophyll components were approximately level with pheophytin *b* and pheophorbide *a*.

Care was needed in the choice of solvent for the pigments when applying them to the cellulose layers. With acetone solutions containing 20 % of water, much chlorophyll remained on the baseline after development, and could have been mistaken for chlorophyllide. This difficulty did not arise with water-free acetone solutions, with which conversion of chlorophylls to pheophytins was also minimised. Water should therefore be removed from acetone extracts of leaves before applying them to the cellulose. This can be done by repeated evaporation at low pressure and room temperature, with addition of further acetone (A.R.) after each distillation, until the residue is dry, or by shaking with ammonium acetate (1 g/5 ml of extract) and diethyl ether (1 vol.) and allowing the phases to separate. The possibility of a similar source of error in paper chromatography and in thin-layer chromatography on other supports must be borne in mind. Preliminary tests with silica gel G indicated that much chlorophyll *a* remained on the baseline when spotted from a water-free solvent rather than from one containing water.

Chromatography on microscope slides

Cellulose layers on microscope slides were found to be suitable for rapid qualitative work. A mixture of cellulose powder and water was applied to the microscope slide and smoothed with a glass rod; the layer was dried horizontally at 105° for 25 min. A small covered cylindrical vessel lined with filter paper was used for development. The R_F values of the pheophorbides and chlorophylls were higher than with large plates run for 15 cm, and the pheophytins were less well separated, particularly

when using the Whatman powder. Even smaller amounts of the pigments were visible, however, and development took less than 5 min.

Quantitative chromatography

The possibility of using thin-layer chromatography on cellulose to estimate mixtures of chlorophylls and pheophytins quantitatively was also investigated. Purified samples of each of the four pigments were dissolved in petroleum spirit–acetone–*n*-propanol (90:10:0.45, v/v/v), and aliquots (80 μl) of the solutions were mixed together. The mixture was applied as a streak about 16 cm long to a 20 cm × 20 cm plate; an applicator suitable for this has been described recently[7]. After development of the chromatogram in the dark, over a distance of 15 cm, the chlorophyll *a* band was scraped off and mixed immediately with acetone (A.R., 10 ml) in a stoppered centrifuge tube. This was repeated as soon as possible with chlorophyll *b*, using 5 ml of acetone, and then with the pheophytins. After gentle shaking for about 1 min, the mixtures were centrifuged and the supernatant fluids were removed by pipette for spectrophotometric estimation. Absorbancies were measured at 700 mμ and at the wavelength of maximum absorption in the red region of the spectrum, and the difference between each pair of figures was noted. Use was made of the absorption maximum in the red region because any carotenoid contamination would affect the absorption in the blue region; the reading at 700 mμ was deducted to allow for light-scattering by cellulose still suspended in the supernatant fluid. Absorbancy differences were measured also on aliquots of the original pure pigment solutions, diluted with acetone but not subjected to chromatography; losses during the chromatographic procedure could then be calculated.

Oxidative bleaching, especially of the chlorophylls, occurs readily and is increased by illumination or by exposure of the pigments on a dry chromatographic surface. It was therefore essential to do all the operations rapidly in the minimum of light, speed being particularly important during removal of the pigment zones from the plate. Reflected light from a partly shaded 25 W tungsten lamp in a dark-room was found satisfactory when illumination was necessary. Table II shows the recoveries obtained under these conditions. In experiments 5, 7 and 9, in which the pheophytins were not included, the area between chlorophyll *a* and the solvent front was scraped off and eluted with acetone to test for the presence of pheophytins. The absorbancy of the eluate was equivalent to 0.5 ($\pm$ 0.4) μg of pheophytin *a* or 0.7 ($\pm$0.6) μg of pheophytin *b*, or to even smaller amounts of a mixture of the two. This suggests that very little chlorophyll is converted to pheophytin during chromatography. Examination in each of the experiments of the spectrum of the chlorophyll *b* eluate indicated that only about 1% of the chlorophyll *a* tailed into the chlorophyll *b* zone.

The method appears to be suitable for quantitative analysis of mixtures of chlorophylls and pheophytins, and perhaps also of pheophorbides and chlorophyllides, although practice in rapid working was necessary before recoveries of chlorophyll *a* were adequate. Alternative methods of estimation have been reviewed recently by HOLDEN[5]. They include the procedure of VERNON[8], who used direct spectrophotometry on plant extracts before and after complete conversion of chlorophylls to pheophytins, and that of TAN AND FRANCIS[9], who obtained excellent recoveries from sugar-starch columns. Comparison with other thin-layer and paper chromatographic methods is difficult, because recoveries are not usually quoted. The cellulose powder

TABLE II

PERCENTAGE RECOVERIES FOR MIXTURES OF CHLOROPHYLLS AND PHEOPHYTINS SUBJECTED TO THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

Experiment No.	*Chlorophyll a*		*Chlorophyll b*		*Pheophytin a*		*Pheophytin b*		*Type of cellulose*
	Load (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	
1	49	93	19	93	—	—	—	—	Pleuger
2	49	89	20	96	—	—	—	—	M. Nagel
3	17	94	7	104	—	—	—	—	Pleuger
4	18	90	7	98	—	—	—	—	Whatman
5	42	91	19	90	—	—	—	—	Pleuger
6	44	90	20	100	24	96	4	97	Pleuger
7	43	89	22	96	—	—	—	—	Whatman
8	46	90	20	96	24	97	4	102	Whatman
9	45	91	25	95	—	—	—	—	M. Nagel
10	44	90	20	88	24	94	4	100	M. Nagel
Averages		91		96		96		100	

used for thin-layer chromatography probably allows sharper separations than would be obtained with paper; thus chromatography under the same conditions as above on Whatman No. 3 paper, the most suitable of several Whatman grades tried, resulted in about 2.5 % of the chlorophyll *a* tailing into the chlorophyll *b* zone.

Acetone was used for elution in preference to diethyl ether because of the volatility and ease of peroxide formation of the latter. To calculate pigment concentrations in acetone, the approximate specific absorption coefficients shown in Table III

TABLE III

APPROXIMATE SPECIFIC ABSORPTION COEFFICIENTS (α) OF CHLOROPHYLLS AND PHEOPHYTINS IN ACETONE (A. R. GRADE, < 1 % OF WATER)

Compound	λ_{max} (red) ($m\mu$)	$\alpha\lambda_{max\ (red)} - \alpha_{700\ m\mu}$ ($l/g \cdot cm$)
Chlorophyll *a*	663	90
Chlorophyll *b*	645	52
Pheophytin *a*	667	53
Pheophytin *b*	654	34

could be used. These values are based on the absorption coefficients in diethyl ether[9–11] and a comparison of the absorptions of the purified pigments in diethyl ether and acetone; on quantitative conversion of the chlorophylls to pheophytins and a comparison of their absorptions in acetone; and on reports by MACKINNEY[12] and VERNON[8]. They are probably not in error by more than 8 l/g·cm.

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SUMMARY

Chlorophylls *a* and *b*, pheophytins *a* and *b*, and pheophorbides *a* and *b* can be separated from each other and from chlorophyllides *a* and *b* on thin layers of cellulose. With precautions, recoveries of chlorophylls and pheophytins exceed 90 %, and it is suggested that the method is suitable for quantitative analysis of mixtures of the pigments. Cellulose layers on microscope slides are useful for rapid qualitative work.

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UNTERSUCHUNGEN ÜBER DIE BEZIEHUNG ZWISCHEN STRUKTUR UND CHROMATOGRAPHISCHEM VERHALTEN IN DER DÜNNSCHICHT-CHROMATOGRAPHIE

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Für die Papierchromatographie postulierte MARTIN[1] eine lineare Beziehung zwischen R_M-Wert[2] und Anzahl homologer Bauelemente. Diese Gesetzmässigkeit konnte in der Folge von vielen Autoren bestätigt werden (für Zusammenfassung siehe Zit. 3, 4).

In der Dünnschichtchromatographie fanden einerseits BRENNER *et al.*[4–6] sowie HALMEKOSKI UND HANNIHAINEN[7] eine Linearität zwischen R_M-Wert und Anzahl CH_2-Gruppen, anderseits beobachteten HROMATKA UND AUE[8] eine lineare Beziehung zwischen log R_F-Wert und Anzahl C-Atome.

Der Beitrag einer bestimmten Gruppe zum R_M-Wert des Gesamtmoleküls kann vom Molekülrest und von der Stellung dieser Gruppe im Molekül abhängig sein. So fanden z.B. SCHAUER UND BULIRSCH[9], dass die Gruppenkonstante (in den folgenden als $G(\mu)$ bezeichnet) für die α- bzw. ε-NH_2-Gruppe im Aminosäuremolekül verschieden ist. Um die gegenseitige Beeinflussung der einzelnen Gruppen zu eliminieren führten LEIBNITZ *et al.*[10] und BEHRENS *et al.*[11] "komplexe Gruppenkonstanten" ein.

Besonders eingehend beschäftigten sich LEDERER[12], BRENNER *et al.*[4–6] und GREEN *et al.*[13,14] mit den von FRANC UND JOKL[15] und von HOWE[16] gefundenen Abweichungen vom Additivitätsprinzip der R_M-Werte. BRENNER *et al.*[4–6] haben gezeigt, dass die Fliessmittelentmischung die Additivität dünnschichtchromatographischer R_M-Werte wesentlich beeinflusst. In einigen Fällen (Adsorptionschromatographie?) konnte die "log-log"-Beziehung von FRANC UND JOKL[15] gefunden werden. Die englischen Autoren[13,14] haben u.a. ausgeführt, dass die Chromatographiertechnik die Linearität der R_M-Werte beeinflusst und haben festgestellt, dass ausser "normalen" Gruppenkonstanten auch konstitutionelle Verhältnisse sowie atomische $G(\mu)$-Parameter berücksichtigt werden müssen (vgl. dazu auch LEIBNITZ *et al.*[10]).

In Fortsetzung früherer Untersuchungen[4,6] chromatographierten wir Aminosäuren und Carbobenzoxy(CbO)-Aminosäuren auf Kieselgel-G-Schichten unter Verwendung von Äthylalkohol–Wasser (7:3, V/V) als Fliessmittel. In diesem Fliessmittel fanden wir früher[4,6] eine lineare Beziehung zwischen R_M-Werte und Anzahl CH_2-Gruppen homologer Aminosäuren. In den Tabellen I und II sind die R_M-Werte angegeben (für experimentelle Einzelheiten vgl. PATAKI[17]).

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TABELLE I

R_M-WERTE* VON CARBOBENZOXY-AMINOSÄUREN

CbO-Aminosäure	*R_M-Wert*	*CbO-Aminosäure*	*R_M-Wert*
Ala	—0.298	Met	—0.368
Asp	—0.070	Phe	—0.399
Arg	+0.017	Pro	—0.308
Glu	—0.149	Ser	—0.317
Gly	—0.288	Tyr	—0.410
ε-CbO-Lys	+0.070	Val	—0.357

* Mittelwerte aus je 6 Einzelbestimmungen auf Kieselgel-G-Schichten. Fliessmittel: Äthylalkohol–Wasser (7:3, V/V) nach PATAKI[17].

TABELLE II

R_M-WERTE* VON AMINOSÄUREN

Aminosäure	*R_M-Wert*	*Aminosäure*	*R_M-Wert*
Ala	+0.194	Met	—0.017
Asp	+0.061	Phe	—0.087
Arg	+1.510	Pro	+0.410
Glu	—0.087	Ser	+0.176
Gly	+0.269	Tyr	—0.122
Lys	+1.690	Val	+0.061

* Mittelwerte aus je 6 Einzelbestimmungen auf Kieselgel-G-Schichten. Fliessmittel: Äthylalkohol–Wasser (7:3, V/V) nach PATAKI[17].

TABELLE III

$G(CH_2)$-PARAMETER

Verbindungspaar	$G(CH_2)_{AS}$*	$G(CH_2)_{CbO-AS}$**
Gly/Ala	—0.075	—0.010
Glu/Asp	—0.148	—0.079

* Aminosäuren.
** CbO-Aminosäuren.

TABELLE IV

VERSCHIEDENE GRUPPENKONSTANTEN IN DER REIHE VON AMINOSÄUREN UND VON CbO-AMINOSÄUREN

	Freie Aminosäure	*CbO-Aminosäure*
$G(\beta$-COOH)	—0.133	+0.228
$G(\gamma$-COOH)	—0.206	+0.159
$G(\beta$-Phenyl)	—0.281	—0.101
$G(\beta$-OH aliph.)	—0.018	—0.019
$G(\beta$-OH arom.)	—0.035	—0.011

Definiert man für den R_M-Wert von Alanin bzw. von CbO-Alanin:

$$R_{M\,\mathrm{Ala}} = R_{M\,\mathrm{Gly}} + G(\mathrm{CH_2})_{\mathrm{AS}} \tag{1}$$

$$R_{M\,\mathrm{CbO\text{-}Ala}} = R_{M\,\mathrm{CbO\text{-}Gly}} + G(\mathrm{CH_2})_{\mathrm{CbO\text{-}AS}} \tag{1a}$$

Hierbei bezeichnen die Indices AS (= Aminosäure) und CbO die Gruppenkonstanten für die CH_2-Gruppe in beiden Verbindungsreihen, so lässt sich aus Gl. (1) und aus Gl. (1a) $G(CH_2)$ für die beiden Reihen bestimmen.

Bemerkenswert ist, dass die $G(CH_2)$-Parameter bei den freien Aminosäuren und bei den CbO-Aminosäuren verschieden sind* (Tabelle III).

Die beiden Verbindungspaare Glutaminsäure/Asparaginsäure und die entsprechenden CbO-Derivate unterscheiden sich ebenfalls durch eine CH_2-Gruppe. Subtraktion beider R_M-Werte ergibt für beide Verbindungsreihen die $G(CH_2)_{\mathrm{Asp/Glu}}$-Parameter, welche von den $G(CH_2)$-Parameter erheblich abweichen (vgl. Tabelle III).

Tabelle IV enthält weitere Gruppenkonstanten, welche in Anlehnung an REICHL[18] und an SCHAUER UND BULIRSCH[9] nach den Gln. (2) bis (6) berechnet wurden:

$$G(\beta\text{-COOH}) = R_{M\,\mathrm{Asp}} - R_{M\,\mathrm{Ala}} \tag{2}$$

$$G(\gamma\text{-COOH}) = R_{M\,\mathrm{Glu}} - R_{M\,\mathrm{Ala}} - G(\mathrm{CH_2})_{\mathrm{AS}} \tag{3}$$

$$G(\beta\text{-OH aliph.}) = R_{M\,\mathrm{Ser}} - R_{M\,\mathrm{Ala}} \tag{4}$$

$$G(\beta\text{-OH arom.}) = R_{M\,\mathrm{Tyr}} - R_{M\,\mathrm{Phe}} \tag{5}$$

$$G(\beta\text{-Phenyl}) = R_{M\,\mathrm{Phe}} - R_{M\,\mathrm{Ala}} \tag{6}$$

(analog für die CbO-Aminosäuren).

Wie bereits erwähnt, zeigen die $G(CH_2)_{\mathrm{Asp/Glu}}$-Parameter von den $G(CH_2)$-Parameter grosse Abweichungen (Tabelle III). Diese Tatsache ist leicht zu erklären. Glutaminsäure und Asparaginsäure unterscheiden sich zwar formal durch eine CH_2-Gruppe, jedoch ist die gegenseitige Lage, und somit auch die gegenseitige Beeinflussung, der beiden Carboxyl-Gruppen in den beiden Verbindungen verschieden. Sie stellen deshalb im Sinne vom MARTIN[1] keine homologe Verbindungen dar.

Für die R_M-Werte von Asparaginsäure, Glutaminsäure und deren CbO-Derivate kann man schreiben:

$$R_{M\,\mathrm{Asp}} = R_{M\,\mathrm{Gly}} + G(\mathrm{CH_2})_{\mathrm{AS}} + G(\beta\text{-COOH})_{\mathrm{AS}} \tag{7}$$

$$R_{M\,\mathrm{Glu}} = R_{M\,\mathrm{Gly}} + 2\,G(\mathrm{CH_2})_{\mathrm{AS}} + G(\gamma\text{-COOH})_{\mathrm{AS}} \tag{8}$$

(analog für die CbO-Verbindungen).

Aus Gl. (7) und Gl. (8) folgt:

$$G(\mathrm{CH_2})_{\mathrm{Asp/Glu}} = R_{M\,\mathrm{Glu}} - R_{M\,\mathrm{Asp}} = G(\mathrm{CH_2})_{\mathrm{AS}} + G(\gamma\text{-COOH})_{\mathrm{AS}} - G(\beta\text{-COOH})_{\mathrm{AS}} \tag{9}$$

$G(CH_2)_{\mathrm{Asp/Glu}}$ ist demnach in beiden Reihen mit einem "Fehler" behaftet.

* Diese Tatsache wiederspricht nicht dem MARTIN'schen-Postulat, da durch die Einführung der CbO-Gruppe auch die Ionisierung der NH_2- und COOH-Gruppe verändert wird.

Aus Gl. (9) folgt:

$$G(CH_2)_{AS} = G(CH_2)_{Asp/Glu} - G(\gamma\text{-COOH})_{AS} + G(\beta\text{-COOH})_{AS} = -0.148 + 0.206 \quad -0.133 = -0.075 \tag{10}$$

und:

$$G(CH_2)_{CbO\text{-}AS} = G(CH_2)_{CbO\text{-}Asp/CbO\text{-}Glu} - G(\gamma\text{-COOH})_{CbO\text{-}AS} + G(\beta\text{-COOH})_{CbO\text{-}AS} = -0.079 - 0.159 + 0.228 = -0.010 \tag{10a}$$

Die nach Gl. (10) und Gl. (10a) berechneten $G(CH_2)$-Parameter stimmen mit den Werten der Tabelle III überein.

Subtrahiert man aus dem R_M-Wert einer CbO-Aminosäure den R_M-Wert der entsprechenden freien Aminosäure, so erhält man den jeweiligen scheinbaren Beitrag der CbO-Gruppe zum R_M-Wert des Moleküls. Tabelle V enthält die G("CbO")-Parameter, die erwartungsgemäss grosse Abweichungen zeigen. Die Differenzen lassen sich folgendermassen deuten.

TABELLE V

G("CbO")-PARAMETER

Verbindungspaar (CbO-Aminosäure/Aminosäure)	*G("CbO")*	*Verbindungspaar (CbO-Aminosäure/Aminosäure)*	*G("CbO")*
Ala	−0.492	Met	−0.351
Asp	−0.131	Phe	−0.312
Arg	−1.493	Pro	−0.718
Glu	−0.062	Ser	−0.493
Gly	−0.557	Tyr	−0.288
Lys	−1.602		

Betrachten wir zunächst die Gruppenkonstante $G(\text{"CbO"})_{Gly}$ bzw. $G(\text{"CbO"})_{Ala}$. Bildet man die Differenzen:

$$G(\text{"CbO"})_{Ala} - G(\text{"CbO"})_{Gly} = +0.065 \tag{11}$$

ferner:

$$G(CH_2)_{Gly/Ala} - G(CH_2)_{CbO\text{-}Gly/CbO\text{-}Ala} = -0.065 \tag{12}$$

so stimmen die rechten Seiten von Gln. (11) und (12) mit umgekehrten Vorzeichen überein; d.h. die Unterschiede der G("CbO")-Parameter entsprechen dem Unterschied der $G(CH_2)$-Parameter in den beiden Reihen.

Ausserdem gilt:

$$G(\text{"CbO"})_{Glu} - G(\text{"CbO"})_{Asp} = +0.069 \tag{13}$$

und:

$$G(CH_2)_{Glu/Asp} - G(CH_2)_{CbO\text{-}Glu/CbO\text{-}Asp} = -0.069 \tag{14}$$

Um die "wahre" Gruppenkonstante für die CbO-Gruppe zu erhalten, muss $G(\text{"CbO"})_{\text{Ala}}$ mit —0.065 korrigiert werden:

$$G(\text{"CbO"})_{\text{Ala}} - 0.065 = -0.492 - 0.065 = -0.557 \quad (15)$$

Für Asparaginsäure erscheint $G(\beta\text{-COOH})_{\text{AS}} - G(\beta\text{-COOH})_{\text{CbO-AS}}$ und für Glutaminsäure $G(\gamma\text{-COOH})_{\text{AS}} - G(\gamma\text{-COOH})_{\text{CbO-AS}}$ als zusätzliches Korrekturglied, d.h.:

$$G(\text{"CbO"})_{\text{Asp}} - 0.069 + G(\beta\text{-COOH})_{\text{AS}} - G(\beta\text{-COOH})_{\text{CbO-AS}} = -0.131 - 0.069 - 0.133 - 0.228 = -0.561 \quad (16)$$

bzw.:

$$G(\text{"CbO"})_{\text{Glu}} - 2 \times (0.069) + G(\gamma\text{-COOH})_{\text{AS}} - G(\gamma\text{-COOH})_{\text{CbO-AS}} = -0.062 - 0.138 - 0.206 - 0.159 = -0.565 \quad (17)$$

Der berechnete Mittelwert aus $G(\text{"CbO"})_{\text{Gly}}$ sowie aus Gln. (15), (16) und (17):

$$G(\text{CbO}) = -0.560 \quad (18)$$

dürfte den "wahren" Beitrag der CbO-Gruppe zum R_M-Wert in guter Näherung angeben.

Die Abweichungen der übrigen $G(\text{"CbO"})$-Parameter der Tabelle V von der "wahren" Gruppenkonstante für die CbO-Gruppe können vermutlich durch Heranziehung weiterer Vergleichssubstanzen auf ähnliche Weise beseitigt werden. In der vorliegenden Arbeit sollte lediglich gezeigt werden, dass der Beitrag einer Gruppe zum R_M-Wert des Gesamtmoleküls von den übrigen Gruppen im Molekül abhängig ist.

ZUSAMMENFASSUNG

Durch Vergleich der R_M-Werte von organischen Verbindungen, die sich um bestimmte Gruppen voneinander unterscheiden, wird gezeigt, dass der Beitrag einer Gruppe zum dünnschichtchromatographischen R_M-Wert des Moleküls von den übrigen Gruppen im Molekül abhängig ist.

SUMMARY

Comparison of the R_M values of organic compounds differing by specific groups shows that in thin-layer chromatography the contribution of one group to the R_M value of the molecule is dependent on the other groups in the molecule.

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THE EFFECT OF THE COMPOSITION OF THE MIXED PHASE UPON R_M COEFFICIENTS IN SOLVENT SYSTEMS OF THE TYPE: NONPOLAR SOLVENT + *n*-PENTANOL/AQUEOUS BUFFER SOLUTION

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The effect of the composition of the phases upon the partition of solutes is not only of practical importance in the choice of optimal conditions for separations, but it can also provide information concerning the partition mechanism and molecular interactions. Thus, the slope of the log K = f(log x) line (K = the partition coefficient; x = the molar fraction of the complexing solvent in the mixed phase) often permits the determination of the formula of the complex formed by the metal ion with the solvent[1]. For solvent systems in which only weaker interaction forces of the dispersion type are involved (simple solutions), KEMULA, BUCHOWSKI AND TEPEREK[2] and BUCHOWSKI[3] have derived equations relating the partition coefficients to the composition of the mixed phase. In another paper[4] these authors determined the excess free enthalpy of mixtures of carbon disulphide and hexadecane, on the basis of the experimental relationship between the partition coefficients and the composition of the mixed phase.

Assuming certain simplifications the R_M value is related to the partition coefficient of the chromatographed solute, and its dependence on the composition of the solvent is then analogous to that of partition coefficient[5]. In a series of studies[5–10] (*cf.* also ref. 11) several R_M *vs.* composition relationships were determined. They were found to be practically linear; however, in most cases investigated the solvent systems did not deviate far from ideal solutions. These investigations suggested that paper chromatographic data may also provide information concerning the deviations from ideality of mixed solvents and the partition mechanism[12]. Chromatographic data are less accurate on comparison with bulk partition methods and are confined to a narrower range of values (R_M can be determined with a sufficient accuracy only in the range −1.0 to +1.0); nevertheless, these disadvantages are compensated by the simplicity of the chromatographic technique, economy in solvents and solute and by the feasibility of serial experiments with several solutes simultaneously.

For moderate departures from ideality (g^E of the order of several tens of calories per mole for an equimolar mixture) the deviation of the R_M *vs.* composition relationship from linearity will lie within the experimental error of the chromatographic method (deviations from linearity are the differences between an experimental curve and a straight line joining the R_M values between 0 and 100% pentanol). According to one of the papers cited[2] the deviation for an equimolar mixture a-

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mounted to, *e.g.*, 50/4.575 $T = 0.04\ R_M$, which corresponds to less than 0.03 R_F units. Deviations of that order have been obtained for the system cyclohexane + benzene/aqueous buffer solution[9]. Also WALDI's data[13] on the systematic analysis of alkaloids in the system cyclohexane + chloroform/formamide and presented as R_M *vs.* composition relationships, are approximately linear in view of the moderate deviations from ideality of the mixed phase.

In the present work the R_M *vs.* composition relationships were determined for several solvent systems in which the mixed (organic) phase was composed of pentanol and a non-polar solvent (decalin, xylene, chlorobenzene, tetralin, or iso-amyl ether). Systems of this type have proved very useful in the separation of alkaloids (see ref. 14).

The investigation had a double purpose:

(1) To find out if the R_M coefficients deviate from linearity for solvent systems in which stronger interactions between the component solvents of the mixed phase occur (in the systems investigated the dilution of pentanol caused its dissociation).

(2) To investigate whether paper chromatographic data may be used for the evaluation of deviations from ideality in mixed solvent systems.

The chromatographed substances were isoquinoline, quinoline and acridine. The pH values of the buffered aqueous phase were chosen so that the R_M values were in the optimal range of -1.0 to $+1.0$.

EXPERIMENTAL

The manufacturer and the degree of purity of the solvents used are listed below:

n-Pentanol—British Drug Houses, Great Britain, laboratory reagent.

m-Xylene—Merck Darmstadt, Germany, redistilled.

Tetralin—Xenon Łódź, Poland, analytical reagent.

Decalin—VEB Laborchemie, Germany, laboratory reagent.

Chlorobenzene—Chem. Plants Oświęcim, Poland, laboratory reagent.

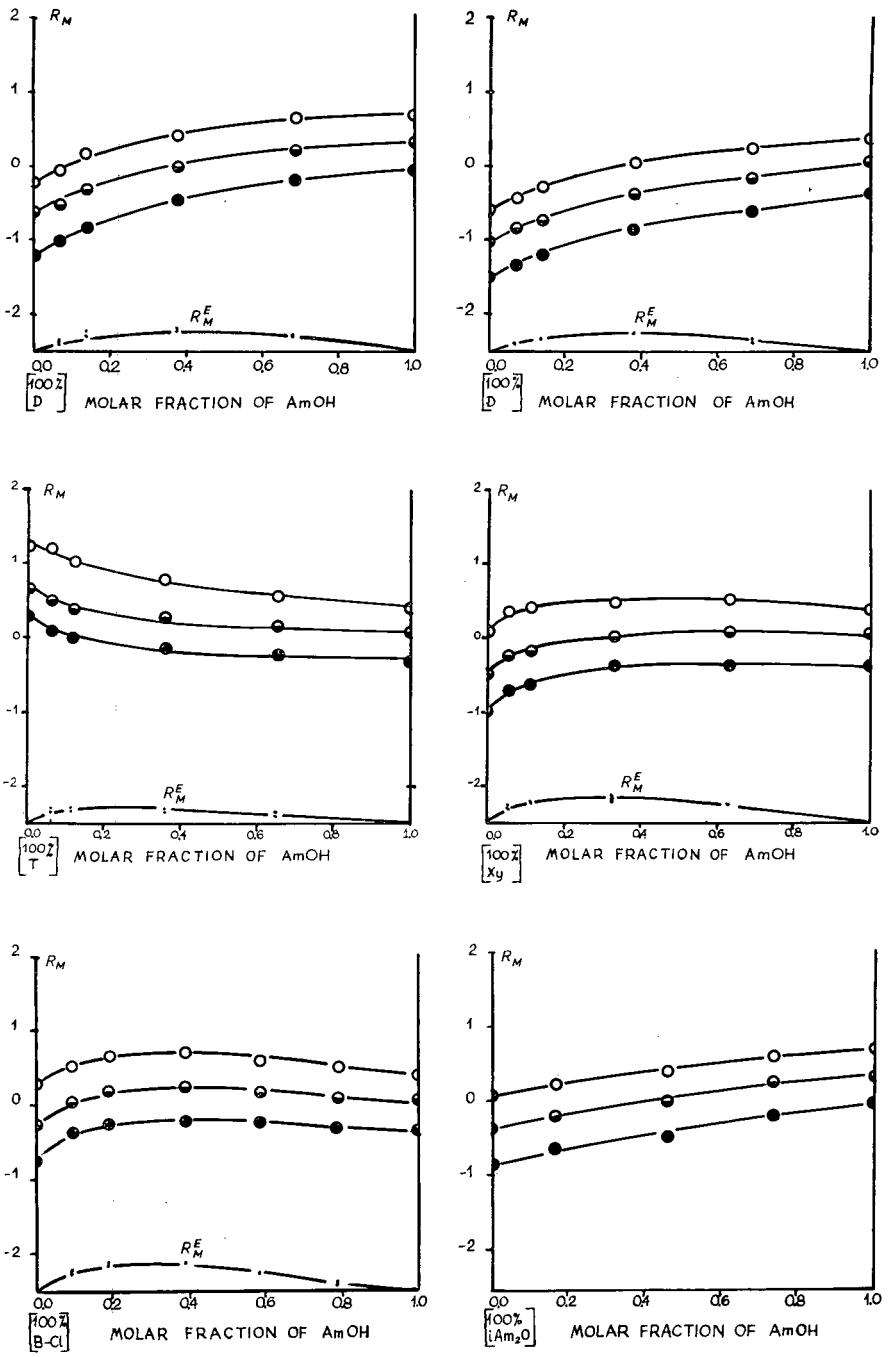
Iso-amyl ether—Reagent Distribution Bureau Gliwice, Poland, laboratory reagent.

The paper strips (Whatman No. 4, 5 × 23.5 cm, cut at right angles to the machine direction) contained 0.5 ml of buffer solution per 1 g of dry paper, which was controlled by weighing (the "moist paper method", *cf.* ref. 15). The strips were impregnated with McIlvaine's buffer solutions: 0.2 *M* Na_2HPO_4 + 0.1 *M* citric acid; the pH was controlled with a Radiometer Copenhagen type PHM 22e valve pH-meter. The glass chromatographic tanks, 6 × 14 × 21 cm, were saturated with the vapours of both phases. The bases were spotted as solutions in benzene (0.5 μl, 0.5 % w/v) and detected with Dragendorff's reagent. The experiments were carried out at room temperature (18.0° ± 1.0°).

RESULTS

The results are presented in Figs. 1–6 as R_M *vs.* molar composition plots. R_M is defined here, as in previous papers, according to REICHL: $R_M = \log[R_F/(1 - R_F)]$.

It can be shown that in the systems studied the R_M coefficient can be expressed on a mol/volume concentration scale (as is usual) instead of in molar fractions (^xR_M derived from ^xk, *cf.* ref. 3). Owing to the comparable molar volumes of the organic



Figs. 1–6. The effect of dilution of pentanol with non-polar solvents on the R_M value. O—O = acridine; ◒—◒ = quinoline; ●—● isoquinoline. Diluting solvents: 1 and 2 = decalin; 3 = tetralin; 4 = *m*-xylene; 5 = chlorobenzene; 6 = iso-amyl ether; $R_M{}^E$ at the bottom denotes deviations of R_M from linearity.

solvents investigated, only the slope of the R_M *vs.* composition curve will be changed; the shape of the curve and its deviations from linearity will remain practically unchanged. For chlorobenzene and xylene, which have molar volumes very similar to that of pentanol, the molar composition scale on the abscissa could be replaced by volume composition.

Except for the tetralin + pentanol/buffer solution (Fig. 3), in all the systems marked positive deviations from linearity of the R_M *vs.* composition relationships are observed. This was to be expected, as mixtures of alcohols with non-polar solvents usually exhibit strong positive deviations from ideality (mixture of solvents of classes II and V, according to the classification proposed by EWELL, HARRISON AND BERG[16]). The deviations of R_M coefficients from ideal values (linearity) amounted to 0.3 R_M units for solvents of class V (up to *ca.* 0.15 R_F units), the maximum deviations occurring at the left hand side of the diagrams, at *ca.* 30 mol % of pentanol. Smaller deviations were observed for mixtures of pentanol with iso-amyl ether (class III according to EWELL *et al.*[16]). The dissociation effect of pentanol was in this case compensated by the formation of hydrogen bonds between the ether and alcohol molecules.

The interpretation of the R_M *vs.* composition relationship for the tetralin + pentanol system is difficult. In this case negative deviations from linearity are observed (Fig. 3), although it is a mixture of the "II + V" type and should exhibit positive deviations, as was the case for the remaining systems. It is perhaps worth noting that the R_M and R_F values of the solutes are higher for tetralin than for pentanol while the reverse is observed for the remaining non-polar solvents. Furthermore, all the systems studied are characterized by strong interactions of the hydrogen bond type between the molecules of the heterocyclic bases and pentanol, and this interaction may influence the magnitude of deviations from linearity.

The deviations from linearity were in a given system identical (within experimental error) for all three heterocyclic bases, which is probably due to the similarity of their molecular structure. The comparison of Figs. 1 and 2 indicates that the pH of the buffer solutions, which controls the R_M values of the organic bases studied, does not influence the magnitude of the deviations. The curves in Fig. 2 are shifted towards a lower range of R_F values; since their shape remains unaltered, this suggests that any gradient effects are negligible in the technique employed, provided that R_F values are in the optimal range 0.1–0.8.

SUMMARY

The relationships between R_M values and composition of the mixed phase were determined for a number of solvent systems of the type: non-polar solvent + *n*-pentanol/buffer solution. The following solvents were mixed with pentanol: decalin, tetralin, *m*-xylene, chlorobenzene and iso-amyl ether. Except for tetralin (negative deviations), marked positive deviations from linearity of the R_M *vs.* molar composition relationship were observed. The deviations did not exceed 0.3 R_M units and the maxima were in the range of lower concentrations of pentanol. The deviations were practically equal for all three chromatographed substances and were caused by dissociation of pentanol due to dilution with the non-polar solvents. The experimental results indicate that chromatographic data may provide information concerning the non-ideality of solvent mixtures.

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THE COLOUR REACTIONS OF THE HYDROXYSKATOLES

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A number of reports have appeared in the literature, in recent years, of relatively specific colour reactions for the various hydroxyindoles, containing the hydroxyl group in the benzene moiety of the indole ring system. For instance, 5-hydroxyindoles have been reported to give violet colours specifically with 1-nitroso-2-naphthol in the presence of nitrous acid[1]. 6-Hydroxyindoles (and 6-alkoxyindoles), with a free 2-position, were said to give immediate bright red colours with diazonium salts under acid conditions[2]. 7-Hydroxyindoles were, by contrast, reported to give slowly forming brownish-purple colours with the acid diazo reagent, and indoles hydroxylated in the 4- and 5-positions were said to merely give weak brown or yellow colours under these conditions[2]. However, as far as the authors are aware, these colour reactions have only been studied with a relatively small number of hydroxyindole derivatives, particularly in the cases of the 4- and 7-hydroxyindole derivatives. A summary of the various colour reactions that have been employed, to date, in the paper chromatography of the hydroxyindoles (mostly 5-hydroxyindole derivatives) is contained in a recent review on the determination of hydroxyindoles in biological materials by SANDLER[3].

There are a number of reports in the literature concerning possible relationships between the urinary excretion of relatively large amounts of "6-hydroxyskatole sulphate" and some forms of mental illness (*cf.* reviews by SPRINCE[4] and RODNIGHT[5]). It has recently been shown, however, by enzymatic hydrolysis and thin-layer chromatography, that the substance previously considered to be "6-sulphatoxyskatole" is, in all probability, a mixture of several different hydroxyskatole conjugates[6]. An essential prerequisite for this investigation was a complete understanding of the chromatographic behaviour of all four isomeric hydroxyskatoles. Several paper and thin-layer chromatographic systems have been developed, in these laboratories, for the separation and identification of the various isomeric hydroxyskatoles[7,8]. A preliminary report of the colours given by the 4-, 5-, 6- and 7-hydroxyskatoles with several different chromogenic reagents has already appeared[7]; this communication will describe a much more extensive survey of the colour reactions of this group of compounds.

EXPERIMENTAL

Hydroxyskatoles

The hydroxyskatoles were prepared by the methods described in the literature[9].

Chromogenic reagents

The preparation of the various chromogenic reagents, used in this investigation,

is described in detail, since it has sometimes been observed that relatively minor changes in the method of preparation of a reagent can result in marked changes in the final colour developed with a particular compound.

(*i*) *Ehrlich's reagent.* A solution of *p*-dimethylaminobenzaldehyde (1 g) in a mixture of concentrated hydrochloric acid (25 ml) and methanol (75 ml).

(*ii*) *Van Urk's reagent.* A solution of *p*-dimethylaminobenzaldehyde (0.125 g) in 100 ml of 65 % sulphuric acid, containing 0.1 ml of 5 % aqueous ferric chloride.

(*iii*) F_1. A solution of 4-N,N-bis-(2-chloroethyl)-aminobenzaldehyde* (1 g) in a mixture of concentrated hydrochloric acid (25 ml) and methanol (75 ml) (*cf.* REIO[10]).

(*iv*) F_2. A solution of 4-N,N-bis-(2-chloroethyl)-amino-2-tolualdehyde* (1 g) in a mixture of concentrated hydrochloric acid (25 ml) and methanol (75 ml) (*cf.* REIO[10]).

(*v*) *DMCA.* A solution of *p*-dimethylaminocinnamaldehyde (2 g) in a mixture of 6 *N* hydrochloric acid (100 ml) and ethanol (100 ml). This solution was diluted with 5 volumes of ethanol immediately prior to use (*cf.* HARLEY-MASON AND ARCHER[11]).

(*vi*) *Terephthalaldehyde reagent.* A solution of terephthalaldehyde (0.2 g) in a mixture of acetone (90 ml) and glacial acetic acid (10 ml). After spraying with this reagent the plates were heated at 105° for 3 min (*cf.* CURZON AND GILTROW[12]).

(*vii*) *Procházka's reagent.* A mixture of 35 % aqueous formaldehyde (10 ml) and 25 % hydrochloric acid (10 ml) diluted with 95 % ethanol (20 ml). After spraying with this reagent the plates were heated at 105° for 5 min (PROCHÁZKA *et al.*[13] and *cf.* RANDERATH[14]).

(*viii*) *Xanthydrol reagent.* A solution of xanthydrol (0.2 g) in a mixture of ethanol (90 ml) and concentrated hydrochloric acid (10 ml) prepared immediately before use (*cf.* DICKMAN AND CROCKETT[15]).

(*ix*) *1-Nitroso-2-naphthol–nitrous acid reagent.* The plate was first sprayed with a 1 % solution of 1-nitroso-2-naphthol in 95 % ethanol; after drying it was sprayed with freshly prepared 2 *N* hydrochloric acid containing 2 vol. % of a 5 % sodium nitrite solution (UDENFRIEND *et al.*[1] and *cf.* JEPSON[16]).

(*x*) *Sodium periodate–picric acid reagent.* A mixture of equal volumes of 10 % aqueous sodium metaperiodate and 3.5 % aqueous picric acid; prepared at 100° to ensure that the reagents remain in solution (*cf.* CADENAS AND DEFERRARI[17]).

(*xi.a*) *Gibb's reagent* (*neutral*). A solution of N,2,6-trichloro-*p*-quinoneimine (2 g) in ethanol (100 ml).

(*xi.b*) *Gibb's reagent* (*basic*). The plate was first sprayed with the neutral reagent, prepared as described in Section (xi.a), and then exposed to ammonia fumes.

(*xii.a*) *Folin and Ciocalteu's reagent* (*neutral*). This reagent was available commercially (B.D.H.) and was diluted with two volumes of water directly prior to use.

(*xii.b*) *Folin and Ciocalteu's reagent* (*basic*). The plate was first sprayed with the dilute commercial reagent, *cf.* Section (xii.a), and then exposed to ammonia fumes.

(*xiii*) *Gold chloride.* The commercially available reagent (Fisher Scientific Co.); a 0.5 % aqueous solution was used directly (*cf.* ERSPAMER AND BORETTI[18]).

(*xiv*) *2,2-Diphenyl-1-picrylhydrazyl.* A solution of 2,2-diphenyl-1-picrylhydrazyl** (1 g) in ethanol (100 ml) (*cf.* HERZMANN AND VENKER[19]).

* Obtained from the Frinton Laboratories, Vineland, N.J., U.S.A.

** Obtained from the Eastman-Kodak Chemical Company, Rochester, N.Y., U.S.A.

(*xv.a*) *NNCD* (*acid*). A solution of 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate* (0.1 g) in dilute aqueous acetic acid (pH = 2) (100 ml) (*cf.* CARTWRIGHT AND ROBERTS[20]).

(*xv.b*) *and* (*xv.c*) *NNCD* (*alkaline*). After spraying with the NNCD reagent, prepared by the method described above, the plates were sprayed with either: (b) 20 % aqueous sodium carbonate solution or (c) 10 % tetraethylammonium hydroxide solution.

(*xvi.a*) *DSA* (*acid*). Ten volumes of a solution of sulphanilic acid (9 g) in concentrated hydrochloric acid (90 ml) and water (900 ml) were mixed, at 0°, with one volume of 5 % aqueous sodium nitrite. After 5 min the excess nitrous acid was destroyed by the addition of excess ammonium sulphamate (*cf.* JEPSON[16]).

(*xvi.b*) *DSA* (*acid*; *in the presence of excess nitrous acid*). A 5 % aquous solution of sodium nitrite (1.5 ml) was added to a solution of sulphanilic acid (1.5 ml, containing 9 g of sulphanilic acid in 90 ml of concentrated hydrochloric acid per litre of water); a further quantity (6 ml) of the aqueous sodium nitrite solution was then added (*cf.* BLOCK *et al.*[21]). The solution was diluted to 50 ml with water after standing at room temperature for 5 min.

(*xvii*) *NBDF*. A solution of *p*-nitrobenzenediazonium fluoroborate** (0.1 g) in dilute aqueous acetic acid (pH = 2) (100 ml).

Colour development

The hydroxyskatoles (10 μg of each isomer)*** were applied, in methanolic solution, to the origin of Silica gel G plates and the thin-layer chromatograms were developed with one of the solvent systems previously described by the authors for the thin-layer chromatography of the hydroxyskatoles, *i.e.* diisopropyl ether or 1,2-dichloroethane–diisopropylamine (6:1) (*cf.* HEACOCK AND MAHON[8]). After drying in a current of air each developed chromatoplate was sprayed with one of the various colour reagents that are mentioned above. The initial colour development and the subsequent changes in the colour of the spots and the backgrounds were carefully noted.

RESULTS AND DISCUSSION

The colours obtained from the hydroxyskatoles by the action of several different chromogenic reagents, after chromatography on Silica gel G using diisopropyl ether as the developing solvent are shown in Table I. The use of the basic solvent system described above (*i.e.* 1,2-dichloroethane–diisopropylamine (6:1)), which, although giving better separations of the four isomeric hydroxyskatoles, can give unsatisfactory results in some cases, since residual traces of this solvent occasionally interfere, quite strikingly, with the colour produced by a particular hydroxyskatole with certain of the chromogenic reagents. This is not always a problem when a strongly

* Obtained from Hopkins and Williams Ltd., Chadwell Heath, Essex, England.

** Obtained from the Eastman-Kodak Chemical Company, Rochester, N.Y., U.S.A.

*** It was found, in practice, that although 10 μg of the hydroxyskatoles usually gave well defined colours with most of the chromogenic reagents described above, more satisfactory results were obtained with the reagents (vi), (vii), (ix) and (x) if 20 μg of the hydroxy compound were used.

acidic spray reagent (*e.g.* Ehrlich's reagent) is used, presumably due to rapid and complete neutralisation of any residual amine on the plate. The colours reported are those observed in transmitted light, the plates being viewed on an X-ray viewing screen illuminated by diffused daylight-type fluorescent light. As would have been expected somewhat different colours were sometimes observed when a different light source (*e.g.* tungsten light or daylight) was used to illuminate the chromatograms or when the chromatograms were viewed in reflected light.

Since the hydroxyskatoles are both indolic and phenolic in character they give strong colours with typical indole reagents, such as the Ehrlich reagent (*i.e.* *p*-dimethylaminobenzaldehyde), and also with phenol reagents, including the diazonium salts, Folin and Ciocalteu's reagent and Gibb's reagent (*i.e.* N,2,6-trichlorobenzoquinoneimine).

One of the most striking colours obtained from a hydroxyskatole with the Ehrlich reagent was the bright greenish blue colour given by the 6-isomer; 5-hydroxyskatole gave a definite violet colour and the other two isomers gave slightly different shades of greyish blue-violet colours with this reagent.

The colours obtained from the hydroxyskatoles with the Van Urk modification of this reagent (*i.e.* *p*-dimethylaminobenzaldehyde in 65 % sulphuric acid, containing a small quantity of ferric chloride) were fairly well differentiated (see Table I). Initially all the hydroxyskatoles gave yellow colours with the Van Urk reagent, however, the edges of the spots rapidly darkened and the peripheral colours slowly spread inwards and in *ca.* 24 h covered the entire area of the spot. 5-Hydroxyskatole eventually gave a distinct stable blue colour with the Van Urk reagent. This reagent has one practical advantage over the simple Ehrlich reagent; the background of the sprayed chromatograms does not discolour on keeping.

Two relatively new reagents, which are chemically similar to the Ehrlich reagent, *i.e.* 4-N,N-bis-(2-chloroethyl)-aminobenzaldehyde (F_1) and 4-N,N-bis-(2-chloroethyl)-amino-2-tolualdehyde (F_2), which have recently been utilised by REIO as location reagents in indole chromatography[10], gave intense and initially relatively well differentiated colours with the hydroxyskatoles. It is interesting to note, however, that both the reagents (F_1) and (F_2) gave a greenish-blue coloured product with the 7-isomer, similar to the bluish green coloured derivatives given by the 6-isomer with the simple Ehrlich reagent. These reagents appear to be very sensitive for detection of the hydroxyskatoles and may prove useful in the future for the detection and assay of other hydroxyindole derivatives.

p-Dimethylaminocinnamaldehyde, introduced by HARLEY-MASON AND ARCHER as a spray reagent for indoles[11], gives vivid blue to grey-violet colours with the compounds under investigation. This reagent is undoubtedly very sensitive, but suffers from a number of disadvantages (*cf.* DURKEE AND SIROS[22]); firstly, the colours are not particularly well differentiated and secondly the background is quite unstable, rapidly acquiring a pink colour and finally turning red-brown. This chromogenic reagent is quite unsuitable to use in cases where basic solvents have been used during the running of the chromatograms. The residual traces of developing solvents appear to interfere with the colour development and in such cases the initial background colour is often too intense for the spray to be of any practical utility.

The terephthalaldehyde reagent rapidly gives a very characteristic stable bright blue colour with 6-hydroxyskatole. The other isomers only react relatively

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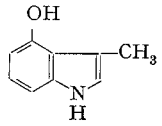
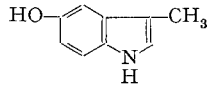
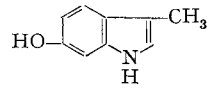
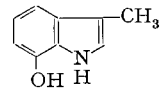
TABLE I

COLOUR REACTIONS OF THE HYDROXYSKATOLES ON SILICA GEL G

Chromogenic reagents	*Colours*[a,b] *given with the hydroxyskatoles*			
	4-Hydroxyskatole 	*5-Hydroxyskatole* 	*6-Hydroxyskatole* 	*7-Hydroxyskatole* 
Ehrlich's reagent	Grey-blue > grey-violet	Violet ⟶ blue-violet	Green ⟶ blue-green	Grey ⟶ blue-grey
Van Urk's reagent[c]	Yellow (grey-brown edge) ⟶ grey-brown	Yellow (blue-grey edge) ⟶ blue	Yellow (green-brown edge) ⟶ olive-green	Yellow (grey-brown edge) ⟶ grey
F_1[d]	Blue	Violet	Blue-violet ⟶ blue	Blue-green
F_2[e]	Grey-blue	Violet	Blue	Blue-green
DMCA[f]	Grey-violet	Blue ⟶ blue-violet ⟶ ⟶ violet	Blue-green ⟶ grey ⟶ ⟶ grey-brown	Violet ⟶ grey-violet
Terephthalaldehyde[g]	Yellow (blue-green edge) ⟶ grey	Weak pink ⟶ orange-pink ⟶ yellow-brown	Blue	Mauve-brown ⟶ red-brown
Procházka's reagent[h]	Dull olive green ⟶ ⟶ grey-brown	Violet-brown ⟶ red-brown	Blue	Grey-violet ⟶ grey-brown
Xanthydrol	Yellow (grey-violet edge) ⟶ grey-green (pink-violet edge)	Pink (blue-violet edge) ⟶ orange (violet edge) ⟶ blue-violet	Yellow-orange (violet edge) ⟶ grey-green (violet edge)	Yellow (violet edge) ⟶ grey-violet
1-Nitroso-2-naphthol–nitrous acid	Dull grey ⟶ grey-green	Grey ⟶ grey-violet ⟶ ⟶ violet	Dull orange-brown	Dull grey-violet
Sodium metaperiodate–picric acid	Dark grey	Brown	Grey-brown	Violet-brown

Gibb's reagent (neutral)	Red-brown ⟶ brown-violet	Violet	Red-brown ⟶ brown	Orange-brown ⟶ orange
Gibb's reagent (basic)	Blue-violet	Blue-violet	Blue-violet	Blue-violet
Folin and Ciocalteu's reagent (neutral)	Grey-blue ⟶ grey	Grey-blue ⟶ grey-violet	Grey-brown ⟶ dull green-brown	Grey-violet ⟶ grey-blue
Folin and Ciocalteu's reagent (basic)	Grey-blue	Grey-blue	Grey-blue	Grey-blue
Gold chloride	Grey-green ⟶ grey	Grey	Greyish olive green	Violet ⟶ grey-violet
2,2-Diphenyl-1-picrylhydrazyl	Pale yellow ⟶ grey-green	Pale yellow ⟶ pink-brown	Pale yellow ⟶ grey-green	Pale yellow ⟶ violet-brown
NNCD[i] (acid)	Red-brown	Orange	Brick red	Orange
NNCD[i] (sodium carbonate)	Grey-brown	Orange	Violet-brown	Yellow-brown
NNCD[i] (tetraethylammonium hydroxide)	Grey-blue	Orange-brown	Blue	Blue-green
DSA[j] (acid; excess nitrous acid removed)	Grey-brown	Yellow-brown ⟶ orange-brown	Pink-brown ⟶ bright red	Violet-brown
DSA[j] (acid; in the presence of nitrous acid)	Orange ⟶ brown	Orange-brown ⟶ brown	Yellow-orange ⟶ yellow-brown	Orange ⟶ brown

[a] The colours reported are those observed by viewing the developed chromatograms (after spraying) in transmitted light on an X-ray viewing screen with diffused fluorescent light (daylight-type) illumination.

[b] The initial colour produced by the particular reagent is reported together with subsequent major colour changes.

[c] In all cases, the hydroxyskatoles initially gave yellow colours with this reagent. However, after a few minutes the final colour began to develop on the periphery of the spot and within 24 h had covered the entire area of the spot.

[d] F_1 = 4-N,N-bis-(2-chloroethyl)-aminobenzaldehyde.

[e] F_2 = 4-N,N-bis-(2-chloroethyl)-amino-2-tolualdehyde.

[f] DMCA = *p*-dimethylaminocinnamaldehyde.

[g] With the exception of the bright blue colour which develops rapidly with the 6-isomer, the colours produced by all the other isomers form very slowly.

[h] There is an apparent marked difference in some of the colours given by the hydroxyskatoles with this reagent on viewing in reflected or transmitted light. The coloured products obtained from the 4- and 6-hydroxyskatoles appear much greener in reflected light.

[i] NNCD = 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate.

[j] DSA = diazotised sulphanilic acid.

slowly with this reagent, particularly the 5-isomer, which initially gives only a very weak pink colour. After 24 h distinct grey and red-brown colours were shown by the 4- and 7-hydroxyskatoles respectively; after about 48 h the spot due to the 5-isomer developed a yellow-brown colour. The colours eventually obtained from the hydroxyskatoles with terephthalaldehyde were quite distinctive in all four cases and the background appears to be quite stable, however, it suffers the disadvantage of being somewhat less sensitive than many of the other reagents tested.

The Procházka reagent (*i.e.* formaldehyde and concentrated hydrochloric acid) gave a definite blue colour with the 6-isomer; the other isomers giving a series of rather dull brownish colours.

The colours obtained from the hydroxyskatoles with the xanthydrol reagent are markedly affected by the presence of residual traces of basic solvents. In general the colours develop slowly with this reagent; under neutral conditions the 4- and 6-isomers initially gave yellow spots (with grey-violet edges); after 24 h the central area of each spot became grey-green in colour, but the violet edge was still apparent. Deep blue-violet and grey-violet spots were eventually obtained from 5- and 7-hydroxyskatole respectively.

The 1-nitroso-2-naphthol–nitrous acid reagent, reported to give violet colours specifically with 5-hydroxyindoles[1], did, in fact, give a violet colour with 5-hydroxyskatole. However, the 7-isomer also gave a dull greyish violet colour with this system, which could be confused, by inexperienced observers, with the colour obtained from the 5-isomer. The other two isomers gave muddy grey to brown colours with this reagent. As reported[1,16] previously, this reagent is not very sensitive and in general, the colours produced were relatively dull. The background colour, which was initially yellow, darkened markedly on standing. (The nitrosonaphthol–nitrite reagent appeared to give brighter colours with the hydroxyskatoles on paper than on silica gel and the background was clearer.)

The sodium metaperiodate–picric acid reagent, initially used for the detection of amines[17], can also be used for the detection of the hydroxyskatoles, although it is relatively insensitive. However, this reagent gave a characteristic strong dark grey colour with the 4-isomer, whilst the other isomers gave dull brown colours with slight grey or violet hues. All the colours were formed on a bright yellow background.

The nature of the colours obtained by the action of Gibb's reagent (*i.e.* N,2,6-trichloroquinoneimine) on the hydroxyskatoles is strongly affected by the presence or absence of traces of bases. Under neutral conditions the 5-isomer gave a distinct violet colour, the other three isomers giving bright red to orange-brown colours. However, traces of base altered these colours in a striking fashion; all the isomers gave blue to grey-violet spots with Gibb's reagent under alkaline conditions. Exposure of the plate to ammonia fumes after, spraying, immediately converted all the spots to a vivid blue-violet colour; spraying with alkaline buffers produced various greyish violet shades. The background rapidly turned brown on exposure to alkali, or if residual traces of basic solvents were present. The alkali sensitivity of these coloured derivatives undoubtedly accounts for the apparent differences in the colours obtained by the action of Gibb's reagent on the hydroxyskatole on paper and on silica as reported in an earlier paper[7]. A neutral running solvent system was used in the case of the separations on formamide paper, whereas a basic solvent system was employed with the silica plates.

Folin and Ciocalteu's reagent gave varying shades of dark grey colours with all four hydroxyskatoles (on a yellow background). Once again, the colours of the products were affected by small quantities of bases; fuming the plates with ammonia, after spraying, converted all the spots to virtually the same grey-blue colour. A quantitative assay procedure for the hydroxyskatoles based on the colours they give with the Folin and Ciocalteu reagent, under alkaline conditions, has recently been described by the authors[23].

Gold chloride gave a distinct violet colour with 7-hydroxyskatole, whilst the other three isomers gave dull grey-green colours with this reagent.

An interesting reagent which has recently been used for the detection of phenols on paper chromatograms is the stable free radical 2,2-diphenyl-1-picrylhydrazyl[19]. Initially the hydroxyskatoles appear as very pale yellow spots on a mauve background when developed chromatograms are sprayed with this reagent. However, after standing for about 24 h at room temperature the spots due to the 4- and 6-isomers develop grey-green colours, whilst the 5- and 7-hydroxyskatoles respectively gave pink-brown and violet-brown derivatives. During this time the initial bright pink-mauve background colour changes to a dull violet-brown.

It has been reported that 6-hydroxy- and 6-alkoxy-indoles react with diazonium salts, under acid conditions, to give brilliant red coloured products[2,16,24]. 6-Hydroxyskatole does not appear to be an exception to this rule, giving a bright red colour with acidified diazonium salts, provided that any excess nitrous acid present in the diazonium salt solution is destroyed before spraying. The DSA reagent (*i.e.* diazotised sulphanilic acid) prepared by the method described by JEPSON[16] gives a bright red colour with 6-hydroxyskatole and grey, orange and violet shades of brown with the 4-, 5- and 7-isomers respectively. However, if the excess nitrous acid is not removed before colour development, merely yellow to orange shades of brown colours are obtained with all four isomers. This accounts for the fact that 6-hydroxyskatole was previously reported by the authors as giving a yellow-brown colour with diazotised sulphanilic acid[7]. The previously reported colours were obtained with diazotised sulphanilic acid in the presence of excess nitrous acid. The reagent was prepared by one of the methods described in BLOCK *et al.*[21].

A number of stabilised diazonium salts are now available commercially, and their use as location reagents in chromatography avoids the necessity of preparing the diazonium salt before use, and eliminates the possibility of excess nitrous acid being present in the reagent and interfering with the colours produced. One of the most useful compounds of this type is the so-called NNCD reagent (*i.e.* 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate). This reagent in dilute acetic acid gave a vivid brick red colour with 6-hydroxyskatole, and although orange colours were obtained from the 5- and 7-isomers, a definite red-brown colour was also obtained from the 4-isomer, which could possibly be confused with the colour obtained from the 6-isomer. The products obtained from the hydroxyskatoles with the NNCD reagent underwent quite striking colour changes on further spraying with aqueous bases (sodium carbonate or tetraethylammonium hydroxide were used). The coloured derivative of the 5-isomer was the least affected, remaining orange on spraying with the sodium carbonate solution and becoming orange-brown on treatment with the stronger alkali. The most striking colour changes were observed with the product derived from the 7-isomer, which turned yellow-brown with the carbonate solution,

TABLE II

COLOUR REACTIONS OF THE HYDROXYSKATOLES ON PAPER

Chromogenic reagents	*Colours given with the hydroxyskatoles with two diazonium salts*			
	4-Hydroxyskatole	*5-Hydroxyskatole*	*6-Hydroxyskatole*	*7-Hydroxyskatole*
NNCD[a] (acid)	Rust	Orange	Brick red	Intense tangerine orange
NNCD/sodium carbonate	Grey-brown	Orange	Dark brown	Olive green
NNCD/tetraethylammonium hydroxide	Grey-blue	Pink ⟶ brown	Bright blue ⟶ grey-blue	Deep turquoise
NBDF (acid)	Brick red	Orange	Red-violet	Orange-red
NBDF/sodium carbonate	Grey-brown	Brown	Violet-brown	Dark brown
NBDF/tetraethylammonium hydroxide	Grey-blue	Grey-brown	Blue ⟶ grey-blue	Blue

[a] NNCD = 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate; NBDF = *p*-nitrobenzenediazonium fluoroborate.

and gave a vivid blue-green colour on spraying with the solution of the strong base. The colour of the product derived from the 6-isomer changed from red to violet-brown on treatment with sodium carbonate, finally turning blue with the strong alkali. This blue product was initially very brightly coloured, however the colour faded quite rapidly, much more rapidly than the corresponding product derived from the 7-isomer. JEPSON *et al.* have reported the formation of green-blue products from 7-hydroxytryptamine and 7-hydroxyindole-3-acetic acid with diazotised *p*-nitroaniline in the presence of sodium carbonate[2]. ACHESON AND HANDS have reported that the 5- and 6-hydroxyskatole give orange and orange-red colours respectively with the Pauly modification of the DSA reagent[25].

The colour reactions of the hydroxyskatoles with diazonium salts and the subsequent colour changes occurring on treatment with alkali were more readily observed on paper, the colours being much brighter than on thin layers of silica gel. Table II lists the colours obtained from the hydroxyskatoles with the NNCD reagent on paper and the subsequent colour changes observed on treatment with alkali. The colours were basically similar to those observed on silica. Analogous coloured products were obtained from the hydroxyskatoles by the use of another commercially available stable diazonium salt (*p*-nitrobenzenediazonium fluoroborate, *i.e.* the NBDF reagent). The results obtained, with this reagent, are recorded in Table II.

CONCLUSIONS

This investigation has shown that there is no shortage of chromogenic reagents for the location of the hydroxyskatoles on developed chromatograms. As would have been expected from previous publications on the colour reactions of hydroxyindole derivatives, 6-hydroxyskatole gave a bright red colour with acid diazo reagents, a deep blue-green colour with the Ehrlich reagent and a violet colour with 1-nitroso-2-naphthol in the presence of nitrous acid.

One of the best colour reagents for 4-hydroxyskatole would appear to be the metaperiodate–picric acid reagent, which gave a distinct dark grey colour with this isomer. The stable blue colour given with the Van Urk reagent and the bright violet colour given with Gibb's reagent (neutral) are two of the most characteristic reactions of the 5-isomer. As well as the distinctive colours given with the acid diazo reagent and Ehrlich's reagent, the stable blue colours given with terephthalaldehyde and the Procházka reagent are very characteristic of the 6-isomer. The most striking colour reaction of the 7-hydroxyskatole was the bright orange obtained with the acid diazo reagents, which changed to a vivid green-blue colour on treatment with strong alkali. The violet colour given with gold chloride also appeared characteristic of the 7-isomer. However, in general, caution should be exercised in deciding the position of the hydroxyl group in a hydroxyindole on the basis of colour reactions alone, since similar colours are often produced with two or more isomers even with some of the most selective reagents.

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SUMMARY

The colour reactions given by 4-, 5-, 6- and 7-hydroxyskatole with a number of different chromogenic reagents are reported. The relative specificity of the reagents for the various positional isomers is discussed.

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KOBALTCHLORID ZUM NACHWEIS PHOSPHORORGANISCHER ESTER AUF PAPIER- UND INSBESONDERE DÜNNSCHICHTCHROMATOGRAMMEN

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Die Notwendigkeit der Identifizierung verschiedenster phosphororganischer Verbindungen auf pflanzlichen Produkten, in forensischem Material, in chemisch-technischen Erzeugnissen sowie in Abwässern und sonstigen industriellen Emissionen lässt es wünschenswert erscheinen, dass für die tägliche Laborpraxis einfache, allgemein übliche Nachweismittel und -methoden ohne apparativen und personellen Aufwand verwendet werden können. Die Papierchromatographie und die Dünnschichtchromatographie bieten hierfür günstige Voraussetzungen.

Während sich die Trennung und der Nachweis schwefelhaltiger phosphororganischer Verbindungen auf Papier- und Dünnschichtchromatogrammen recht befriedigend gestalten lässt, führten die Bemühungen um den Nachweis *schwefelfreier* phosphororganischer Verbindungen, insbesondere der tertiären Ester, bislang zu keinem befriedigendem Ergebnis. Sämtliche der vorgeschlagenen Reagenzien und Verfahren bedingten eine aufwendige, meist mehrstufige Prozedur mit oftmals vieldeutiger Aussage. Hier sei nur soviel erwähnt, dass zahlreiche Autoren sich auf das von HANES UND ISHERWOOD zurückgehende Verfahren[1] der Überführung phosphororganischer Ester in Ammoniumphosphormolybdat und dessen Reduktion zum Molybdänblau stützten und es vor allem hinsichtlich der Art und Mittel des Reduktionsvorganges variierten.

Daneben sind insbesondere für saure Phosphorsäureester folgende Nachweise als geeignet empfohlen worden: das Besprühen mit Fe(III)-salz-Lösungen und nachfolgender Anfärbung mit Sulfosalicylsäure[2], Rhodanidlösung[3] oder 2-*o*-Hydroxyphenyl-benzoxazol[4], ferner das Besprühen mit Bleiacetat- und nachfolgend Sulfidlösung[5] oder mit alkoholischer Silbernitratlösung[6].

Da die dünnschichtchromatographische Technik die Anwendung aggressiver Sprühmittel erlaubt, benutzte BRAUN zur Identifizierung phosphororganischer Weichmacher Antimonpentachlorid[7] und zum Nachweis von Triarylphosphaten wurden Bichromat–Schwefelsäure und Kaliumpermanganat–Schwefelsäure-Mischungen empfohlen[8].

Während die sofortige und direkte Anfärbung der Trialkylphosphate auf Papierchromatogrammen bisher nicht gelang, konnte ein dünnschichtchromatographischer Nachweis dieser Trialkylphosphate von KLEMENT UND WILD[9] durch Besprühen mit einer Lösung von Ammonmolybdat und Perchlorsäure, anschliessendes Trocknen bei 100° und Reduzieren im U.V.-Licht (bei mehrmaliger Wiederholung dieser Prozedur) erreicht werden.

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In einer kürzlich von uns veröffentlichten vorläufigen Mitteilung haben wir über eine neue, einfache Nachweismethode für schwefelfreie phosphororganische Ester berichtet[10]. Durch Ausnutzung der Komplexbildungstendenz zahlreicher phosphororganischer Verbindungen konnte ein Nachweis auf Papier- und Dünnschichtchromatogrammen ermöglicht werden, der nur das Besprühen dieser Chromatogramme mit acetonischer Kobaltchloridlösung notwendig macht. Nachfolgend möchten wir über unsere Versuchsergebnisse ausführlicher berichten und hierbei einige Vergleiche mit anderen Sprühmitteln anstellen sowie auf die Abhängigkeit der Nachweisempfindlichkeit vom Adsorbens und auf geeignete Fliessmittelgemische hinweisen*. Unsere Untersuchungen über die Papier- und Dünnschichtchromatographie phosphororganischer Verbindungen werden fortgesetzt.

EXPERIMENTELLER TEIL

1. Vergleich verschiedener Sprühmittel

Zunächst interessierten wir uns für das schon erwähnte Sprühmittel von KLEMENT UND WILD. Mit diesem Sprühmittel erhält man bessere Resultate, wenn die Konzentration an Perchlorsäure von 4 % auf 10 % erhöht und gleichzeitig die Trockentemperatur auf 150° gesteigert wird. Gute Ergebnisse erhält man auch, wenn man das Sprühmittel vorher im Verhältnis 1:1 mit dem von PETSCHIK UND STEGER[11] beschriebenen Perchlorsäure–Perjodsäure-Gemisch mischt und nach dem Besprühen bei 170° trocknet.

Unsere Versuche zeigten, dass Eisen(III)-salze mit Trialkylphosphaten bei Mengen oberhalb 1 mg gut erkennbare hellbraune Flecken geben. Eine wesentliche Steigerung der Empfindlichkeit durch nachträgliches Besprühen der Dünnschichtplatte mit einer Rhodanid- oder Salicylsäurelösung war jedoch nicht zu beobachten.

Wir dehnten unsere Versuche auch auf andere Metallsalze aus und erzielten so z.B. mit methanolischer Chrom(III)-chloridlösung graugrüne Flecke, die jedoch noch schwächer ausgeprägt waren als die von Eisen(III)-salzlösungen; hellbraune Flecke erhielten wir beim Besprühen der Platten mit einer 1 %igen acetonischen Kupferchloridlösung.

Die mit Abstand günstigsten Resultate sind jedoch beim Besprühen mit einer 1 %igen acetonischen Lösung von Kobaltchlorid (wasserfrei) zu erzielen. Nach dem Verdunsten des Lösungsmittels zeigen sich blaue Flecke, die bereits in der Kälte (bei kleinen Mengen phosphororganischer Ester nach Erwärmen auf 40–50°) innerhalb von 1–2 Min. sichtbar werden. Wasserhaltige Kobaltchloridlösungen erfordern ein nachträgliches Trocknen der Dünnschichtplatte im Trockenschrank; allerdings sind die hierbei nach dem Abkühlen sich abzeichnenden Farbflecke diffus bzw. unscharf gezeichnet.

Mit dem angegebenen Kobaltreagenz können schwefelfreie Phosphorsäureester, Phosphinoxide und Phosphorsäureesterhalogenide als blaue Flecken nachgewiesen werden. Phosphine und Phosphite geben blaue bis blaugrüne Flecke. Die sauren

* Nach Abschluss der hier veröffentlichten Arbeiten wurden uns Versuche von Herrn Dr. H. ACKERMANN (Institut für Ernährungsforschung der DAW, Potsdam-Rehbrücke) bekannt, die dieser zur Identifizierung phosphororganischer Ester mit acetonischer $KMnO_4$-Lösung vorgenommen hat (demnächst in dieser Zeitschrift). Für Trialkylphosphate ist diese Methode nicht geeignet, sie stellt aber eine wertvolle Ergänzung für die Analytik zahlreicher anderer Phosphorsäureester dar.

organischen Phosphate und Phosphite treten nach dem Erwärmen der Dünnschichtplatte auf 60–100° als weisse Flecken auf hellblauem Grund in Erscheinung.

Schliesslich sei noch erwähnt, dass das von MÜHLMANN UND TIETZ[12] für Thiophosphorsäureester angegebene Sprühmittel Kaliumhexajodplatinat mit Trialkylphosphaten nur schwach reagiert, dafür aber zum Nachweis der Triarylphosphate besser geeignet ist als Kobaltchlorid, welches in diesem Falle nur blassblaue Flecke bildet.

2. Abhängigkeit der Nachweisgrenze vom Adsorbens

Wir fanden, dass die Empfindlichkeit des Nachweises mit Kobaltchlorid als Sprühmittel nicht unwesentlich vom Adsorbens abhängt (Tabelle I).

TABELLE I

NACHWEISGRENZE (IN μg) IN ABHÄNGIGKEIT VOM VERWENDETEN ADSORBENS

Substanz	*Adsorbens**		
	I	*II*	*III*
Triäthylphosphat	100	100	50
Tributylphosphat	200	250	80
Triphenylphosphat	250	250	100
Triäthylphosphit	25	10	10
Triamylphosphit	100	100	100
Tributylphosphin	50	25	10
Tributylphosphinoxid	25	25	50
Diisopropylphosphat	10	8	10

* I: Kieselgel D, Charge 30701, VEB Chemiewerk Greiz-Dölau.
II: Kieselgel G, Fa. Merck AG.
III: Aluminiumoxid G, VEB Chemiewerk Greiz-Dölau.

Hierbei wurden verwendet: Kieselgel D, Charge 30701, vom VEB Chemiewerk Greiz-Dölau (Adsorbens I), Kieselgel G der Fa. Merck AG (Adsorbens II) sowie Aluminiumoxid G vom VEB Chemiewerk Greiz-Dölau (Adsorbens III).

Die Nachweisempfindlichkeit auf Papierchromatogrammen entspricht der von Kieselgel D (Adsorbens I).

3. Zur Auswahl der geeignetsten Laufmittel

Das von KLEMENT UND WILD[9] ermittelte Laufmittel Hexan–Benzol–Methanol (2:1:1) liefert im allgemeinen günstige R_F-Werte, jedoch zeigen sich bei den homologen Reihen keine grossen Unterschiede. Unsere Versuche ergaben, dass das Gemisch Hexan–Methanol–Äther (6:1:1) günstige Trennungen zulässt. Beide Laufmittel ergänzen sich in ihren Trenneigenschaften und sie werden nachfolgend als Laufmittel 1 und 2 bezeichnet.

Die für die einzelnen Substanzen mit Laufmittel 1 und 2 gefundenen R_F-Werte sind in Tabelle II (Kieselgel D, Greiz-Dölau) und in Tabelle III (Kieselgel G, Merck) zusammengestellt. Die jeweils aufgetragene Substanzmenge betrug angenähert 2.5 μl. Feste Substanzen sind als 10%ige Lösungen in Methylenchlorid verwendet worden.

TABELLE II

R_F WERTE VON PHOSPHORORGANISCHER VERBINDUNGEN (MIT KIESELGEL D, GREIZ-DÖLAU)

Substanz	*Laufmittel*	
	1	*2*
Trimethylphosphat	0.33	0.05
Triäthylphosphat	0.48	0.21
Triisopropylphosphat	0.52	0.39
Triisobutylphosphat	0.58	0.50
Tributylphosphat	0.61	0.53
Trichloräthylphosphat	0.33	Start
Dichlorvinylphosphorsäuredimethylester	0.48	0.21
Diisopropylfluorphosphat	0.53	0.51
Diisopropylphosphat	0.23	0.04
Methylphosphonsäurediisopropylester	0.42	0.31
Benzylphosphorsäurediäthylester	0.39	0.12
Triphenylphosphat	0.48	0.34
Trikresylphosphat	0.44	0.36
Phenylphosphorsäuredichlorid	0.13	Start
Diäthylaminophosphorsäuredichlorid	0.57	0.50
Triäthylphosphit	0.64	0.67
Triisopropylphosphit	0.71	0.71
Triisobutylphosphit	0.71	0.76
Tributylphosphit	0.72	0.77
Triisoamylphosphit	0.68	0.68
Triamylphosphit	0.68	0.70
Diäthylphosphit	0.33	Start
Dipropylphosphit	0.33	0.04
Dibutylphosphit	0.33	0.12
Tributylphosphin	0.72	0.74
Triphenylphosphin	0.71	0.71
Äthyl-Dipterex	0.29	0.06
Propyl-Dipterex	0.34	0.19
Butyl-Dipterex	0.46	0.31
Tributylphosphinoxid	0.46	0.25

TABELLE III

R_F-WERTE VON PHOSPHORORGANISCHER VERBINDUNGEN (MIT KIESELGEL G, MERCK)

Substanz	*Laufmittel*	
	1	*2*
Trimethylphosphat	—	0.03
Triäthylphosphat	0.45	0.17
Triisopropylphosphat	—	0.35
Tributylphosphat	—	0.39
Methylphosphonsäurediisopropylester	—	0.30
Diisopropylphosphat	0.32	0.01
Benzylphosphorsäurediäthylester	—	0.16
Triphenylphosphat	—	0.23
Triäthylphosphit	—	0.65
Triisobutylphosphit	—	0.68
Di-*n*-propylphosphit	—	0.03
Tributylphosphin	—	0.61
Triphenylphosphin	0.78	—

Mit den angeführten Laufmitteln gelang es z.B., saure Dialkylphosphate neben Dialkylhalogenphosphaten nachzuweisen. Auf die gleiche Weise ist auch die Trennung homologer phosphororganischer Verbindungen möglich, wie dies aus der Fig. 1 am Beispiel der Trialkylphosphate gut ersichtlich ist.

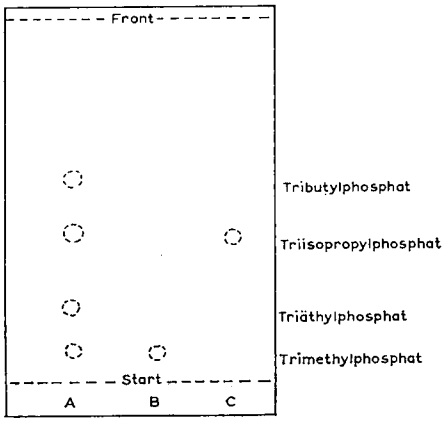

Fig. 1. Trennung von Trialkylphosphaten, aufgetragene Menge 0.005 ml. (A) Jeweils 10% Trimethyl-, Triäthyl-, Triisopropyl- und Tributylphosphat in Methylenchlorid gelöst. (B) 10%ige Lösung von Trimethylphosphat in Methylenchlorid. (C) 10%ige Lösung von Triisopropylphosphat in Methylenchlorid.

ZUSAMMENFASSUNG

Es wird über die Verwendung acetonischer Kobaltchloridlösung zur Identifizierung phosphororganischer Verbindungen auf Papier- und Dünnschichtchromatogrammen berichtet. Auf Vergleiche mit anderen Sprühmitteln, auf die Abhängigkeit der Nachweisgrenze vom Adsorbens und auf geeignete Laufmittel wird näher eingegangen. Die Verwendung der acetonischen Kobaltchloridlösung erlaubt erstmals auch die schnelle und sichere Identifizierung der bisher schwer nachweisbaren Trialkylphosphate.

SUMMARY

A solution of cobalt chloride in acetone is used for the identification of organic phosphorus compounds, both on paper and thin-layer chromatograms. This reagent is compared with other reagents and the dependence of the limits of detection on the adsorbent, as well as the choice of suitable eluents are discussed in detail. The use of this solution also enables a quick and reliable identification of trialkyl phosphates, which have hitherto been difficult to identify.

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A LIQUID SCINTILLATOR-BASED CONTINUOUS RADIO CHROMATOGRAM SCANNER

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INTRODUCTION

ROUCAYROL *et al.*[1] first adapted the principle of liquid scintillation measurement of radio-activity to the direct and continuous scanning of paper chromatograms carrying compounds labelled with isotopes which emit soft β-radiation. In spite of the considerable effort devoted in recent years to techniques for measuring soft β-activity on paper chromatograms this particular method appears to have received little further attention.

In general, the methods adopted for assaying radio-activity distributed in paper chromatograms fall into two classes. Continuous and direct scanning devices utilise either thin-window or windowless Geiger tubes. The efficiency of some of these devices is enhanced by the use of two coaxially mounted Geiger tubes and by a close approach to 4π geometry. A recently introduced instrument in this class was described by SHIPOTOFSKY[2], who also gives a survey of the relevant literature.

The second class of methods embraces all the techniques based upon the use of a scintillator which translates the energy of β-particles into light quanta. With the exception of the method of SELIGER AND AGRANOFF[3] and a recently introduced commercial instrument*, in which anthracene crystals serve as scintillators, the majority employ liquid scintillators. As conceived originally, the distribution of radio-activity was assayed by cutting the developed paper chromatograms into strips at right angles to the direction of solvent flow. These strips were then impregnated with the scintillator solution and individually placed upon the photomultiplier tube. A reflector of aluminium foil placed upon the strip enhanced the light output (SELIGER AND AGRANOFF[3], FUNT AND HETHERINGTON[4]). This technique was developed further by WANG AND JONES[5], who counted the activity of individual strips by immersing these entirely in liquid scintillator contained in a glass vial. The popularity of this technique grew with the introduction of automatic sample changing devices. It has been repeatedly discussed in the literature (LOFTFIELD AND EIGNER[6], SHERMAN[7] and BAXTER AND SENONER[8]).

With the notable exception of the instrument designed by ROUCAYROL *et al.*[1], none of the methods of liquid scintillation counting of paper chromatograms reported in literature permit continuous as well as direct scanning.

The instrument and method of scanning paper chromatograms developed in this laboratory takes the idea of ROUCAYROL *et al.*[1] a stage further. With the aid of

* Panax Ltd., Mitcham, Surrey, Great Britain.

improved geometry, "light-piping" and a more efficient liquid scintillator the sensitivity of the method was enhanced. The method has now been giving satisfactory service for about 2 years.

EXPERIMENTAL

Apparatus and method

The basic design of the instrument is shown in Figs. 1 and 2. Developed paper chromatograms were cut into longitudinal strips, 2.5 cm wide, containing the labelled material. The strips were impregnated with liquid scintillator and mounted on the polished aluminium drum by threading the ends of the strip through a slot and clamping with the spiked and sprung clamp against the inside of the drum.

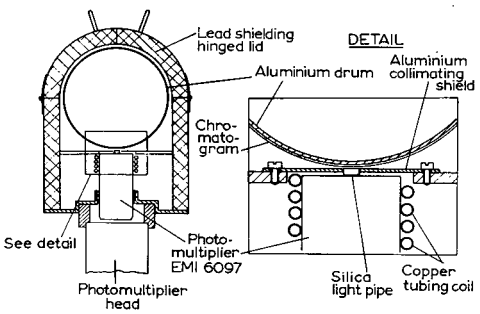

Fig. 1. Diagram of the instrument showing the method of assembly.

The light emitted by the scintillator passed from the chromatogram through a silica prism into the photo-cathode of the photomultiplier tube (E.M.I., type 6097). The silica prism ("Spectrosil" B-quality plate, made by the Thermal Syndicate Ltd., Wallsend, Northumberland, Great Britain), 26.00 mm × 8.00 mm × 3.90 mm, had its horizontal surfaces ground to a smooth finish and its vertical faces polished flat to $^1/_4$ fringe. It functions as an efficient "light pipe" and completely fills the collimating slit (8.0 mm wide). Optical contact between the silica prism and the photomultiplier tube was enhanced with a drop of silicone oil placed between them.

The signals from the photomultiplier tube passed through single channel electronics to a ratemeter and thence to a chart recorder.

The clockmotor drive of the scanner drum advanced the paper chromatogram past the collimating slit at the rate of 30 cm/h. The gear ratio of the chart drive of the recorder was selected to match this speed closely. No special effort was made, however, to synchronise the two movements absolutely.

The aluminium drum and photomultiplier assembly of the scanner were surrounded by a lead shield 5 cm thick. The photo-cathode was cooled by mains water passing through a coil of copper tubing surrounding the upper half of the photomultiplier tube. Although this heat exchanging device is not in direct contact with the photomultiplier tube, enough heat passes from the photo-cathode to the aluminium plate which contains the collimating slit and hence through the metal part of the scanner to the cooling coil, to reduce the background count to about 1.2 to 1.5 c.p.s.

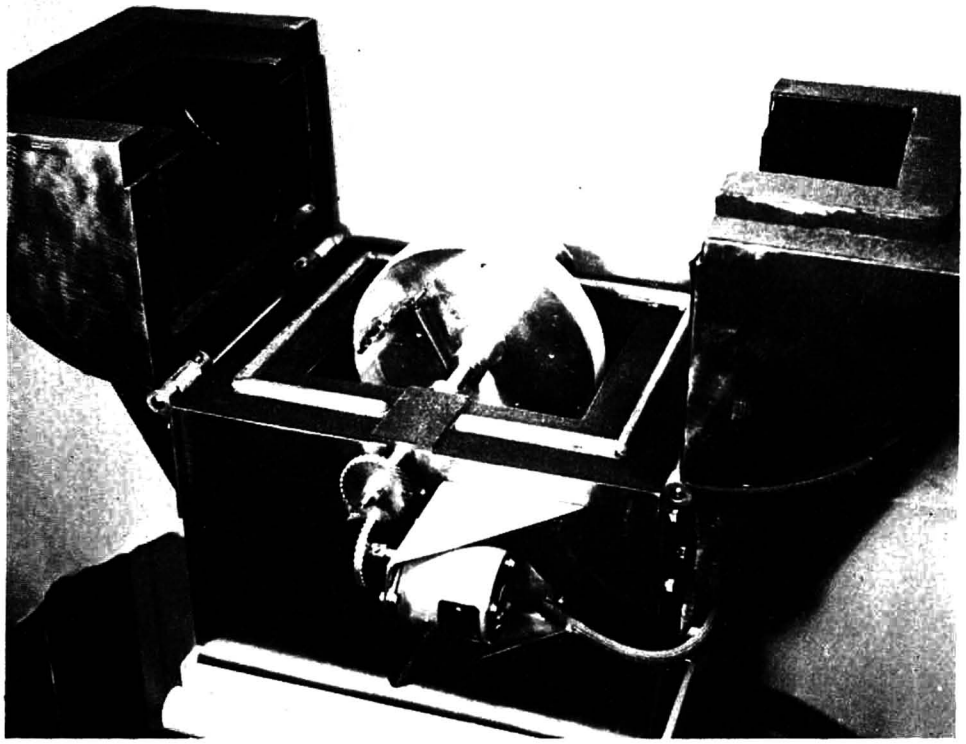

Fig. 2. View of instrument with open lids to show mounting of chromatograms.

This result was achieved with a mains water temperature of about $+10^{\circ}$. No significant lowering of the background count rate was observed by pumping through the cooling coil water chilled to about $+2^{\circ}$.

Materials

The liquid scintillator was purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh, Great Britain. D-Glucose-^{14}C (U) and D-ribose-^{14}C (U) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, Great Britain. Chromatography solvents were made up from laboratory grade materials which were not further purified prior to use. Whatman chromatography papers were used throughout.

Results

All figures stem from scans obtained on chromatograms developed for 24 h by the descending method. In no case has the scanned radioactive material been artificially confined to a conveniently small area on the paper.

The record of a scan (Fig. 3) of a chromatogram carrying five carboxyl-^{14}C labelled amino acids represents the performance of the technique near the limit of its sensitivity.

To check the linearity of the response for different levels of specific activity a

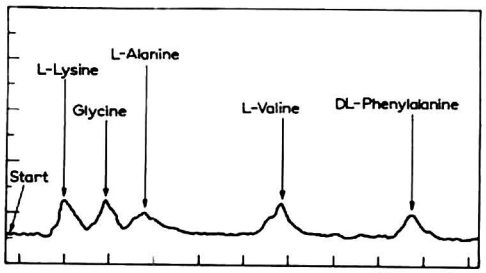

Fig. 3. Record of a chromatogram of 5 amino acids labelled in the carboxyl function with carbon-14. The chromatogram was developed on Whatman No. 3MM paper by descending *n*-butanol–acetic acid–water (4:1:1, v/v) for 24 h. Each amino acid (10 μg) was applied to the starting line in aqueous solution (10 μl) containing 0.1 mμC carbon-14 activity.

series of chromatograms was developed all containing D-glucose (10 μg) and D-ribose (10 μg). The weight (mg) of the peak area on the chart record of each chromatogram was taken as a direct measure of the response of the instrument to the amount of activity present in each "spot". The results are shown in Fig. 4.

Parallel chromatograms carrying unlabelled D-glucose (10 μg) and D-ribose (10 μg) were developed under identical conditions and sprayed with the silver nitrate reagent of TREVELYAN *et al.*[9] to determine the distance from the starting line

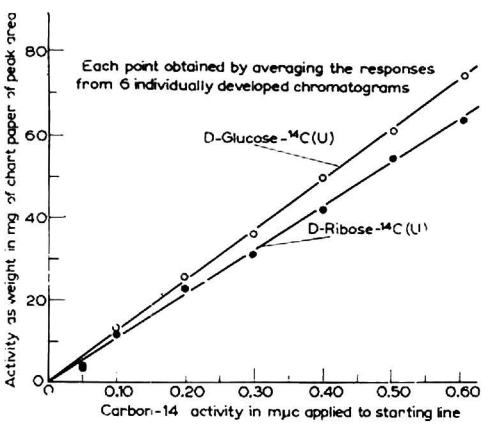

Fig. 4. Relationship between applied activity and response. The standard deviation of each "response" varied from $\pm$ 1.2 to $\pm$ 3.0 mg.

covered by the sugars and the area of the "spots" after 24 h' run when the chromatograms were removed from the tanks and scanned. In no case did the spots extend beyond the width of the strip (2.5 cm) cut from the chromatograms for scanning. D-Glucose travelled a distance of 8.1 cm and spread to cover an area of 6.5 cm^2. The analogous figures for D-ribose were 15.4 cm and 5.9 cm^2, respectively.

The results shown in Table I were produced to investigate the effect which thickness and texture of chromatography papers may have upon the response of the scanner to identical amounts of radio-activity. The peaks on the chart records were cut out and their weight taken as a measure of the response in the same manner in which the results shown in Fig. 4 were obtained.

TABLE I

EFFECT OF THICKNESS AND TEXTURE OF CHROMATOGRAPHY PAPERS UPON THE EFFICIENCY OF THE SCANNING METHOD

Chromatography papers	*Response to* D-*Glucose-*^{14}C *(U) (10 μg, 0.5 mμC). Weight of peak area ± standard deviation (mg)*
Whatman No. 1	50.1 ± 2.1
Whatman No. 2	49.9 ± 1.8
Whatman No. 3	54.2 ± 1.9
Whatman No. 3MM	53.8 ± 2.2
Whatman No. 54	46.6 ± 1.0

All chromatograms were developed for 24 h by descending *n*-butanol–acetic acid–water (4:1:1, v/v).

Each figure was obtained by averaging the response recorded on four identical paper chromatograms.

DISCUSSION

Earlier attempts made in this laboratory to find an efficient method of scintillator-scanning of radio paper chromatograms were based on the use of a solid, plastic scintillator. In this plastic material the scintillating compounds are dispersed in a matrix of polyvinyltoluene (NE 102 plastic scintillator, Nuclear Enterprises (G.B.) Ltd.). The geometry and the mechanical parts of the instrument were similar (Figs. 1 and 2), the plastic scintillator being machined to the same dimensions as the silica prism and occupying the same position in the collimating slit. The performance of this instrument was, however, inadequate. The prospects of enhancing the sensitivity of the instrument were improved with the introduction of a monoisopropylbiphenyl-based liquid scintillator. Compared with toluene or xylene, this solvent has a much lower volatility. It also confers upon the liquid scintillator a significant degree of immunity from quenching by dissolved oxygen (BUCK AND SWANK[10]). The latter property is of particular value in this work, since impregnated paper chromatograms present a relatively large surface area to air.

In paper chromatograms carrying compounds labelled with emitters of soft β-radiation a proportion of the electrons emitted by the decaying nuclides will become absorbed within the paper and will, therefore, fail to interact with any detector of radiation present outside the paper. The presence in the paper of the scintillator solvent in directly impregnated paper chromatograms largely attenuates this limitation (SELIGER AND AGRANOFF[3].) Furthermore, the organic solvent renders the paper relatively translucent (FUNT AND HETHERINGTON[4]).

The instrument was developed to its present state by replacing the originally used polyvinyltoluene "light pipe" with the silica prism in the collimating slit (Fig. 1).

Efficiency

It is difficult to give an absolute assessment of this parameter in paper chromatographic work. However, an idea of the efficiency of this technique may be gained from the results shown in Fig. 3. At the selected settings of the chart recorder full-

scale deflection corresponds to 10 d.p.s. (including background noise). The maximum net amplitude recorded in the amino acid peaks varies from 0.6–1.2 d.p.s. Since the amount of radio-activity of the ^{14}C label of each amino acid amounts to 3.7 d.p.s. (0.1 mμC), it follows that in scanning this particular chromatogram the instrument functioned with an efficiency of from 15 to 30 % at least. This degree of efficiency was maintained throughout and is of practical importance since after 24 h' developing 10 μg of any individual compound may spread to occupy an area of 5 cm^2 or more.

The results presented in Fig. 4 indicate that this method is capable of measuring the distribution of radio-activity in chromatograms with a satisfactory linearity of response. The lower slope recorded for the D-ribose-^{14}C (U) "spots" is due to carbon-14 labelled impurities present in the sample. This sample was about 2 years old at the time and the impurities may have resulted from radiolytic decomposition. A freeze-dried aliquot of an aqueous solution of this sample of carbon-14 labelled D-ribose was assayed in the "Packard" liquid scintillation spectrometer. The results showed that about 20 % of the nominal activity of this sample were volatile and were removed by freeze-drying. The difference between the two slopes in Fig. 4 suggests that the specific activity of D-ribose-^{14}C (U) used in the experiment was about 14 % below that of the D-glucose-^{14}C (U).

The results obtained on chromatograms carrying only 0.05 mμC carbon-14 in each sugar indicate that the efficiency of this method begins to falter under these conditions (area of glucose spot: 6.5 cm^2, area of ribose spot: 5.9 cm^2). The efficiency of the technique, even at these low activities could be improved by slower scanning, since all the scans reported above have been obtained at the relatively high scanning rate of 30 cm/h. A further improvement would result from subjecting the signals from the photomultiplier head to an upper as well as a lower discriminator limit.

Effect of the properties of chromatographic papers

The results in Table I show how the response to identical quantities of D-glucose-^{14}C (U) varies with papers of different thickness and texture. None of the results was significantly different ($P > 0.05$) from the response recorded on Whatman No. 1 paper. The practical usefulness of this method is borne out, however, by the response recorded on chromatograms run in the thick Whatman No. 3 and 3MM papers. In these, the response was slightly higher. It was significantly higher than the result obtained on Whatman No. 54 paper. This suggests that the efficiency of the technique depends upon the amount of liquid scintillator available in the immediate vicinity of the radio-active material in the chromatogram and thus tends to be enhanced, rather than impeded, in thick papers.

ACKNOWLEDGEMENTS

The author is grateful to Mrs. ANN WHITEHEAD and Mr. R. MOULE for skilful technical assistance, to Mr. M. F. JONES for the detailed design of the instrument and to Mr. M. CHETWOOD and Mr. I. COX for its manufacture.

SUMMARY

An instrument for the direct and continuous scanning of paper chromatograms

carrying soft-β emitters has been described. The method of detection is based upon a liquid scintillator with which the developed paper chromatograms are directly impregnated. The response is sufficiently linear to permit direct quantitative assay of radio-activity down to at least 0.1 mμC of carbon-14 per spot of 5 cm^2 or more surface area and is not attenuated in thick papers.

While retaining the advantages of liquid scintillation techniques in general, this method is free from the labour involved in cutting up developed paper chromatograms for counting in glass vials, nor is it subject to the error inherent in the variable geometry of orientation of the strips floating in the liquid scintillator.

The design of the mechanical parts of the scanner reduces to a minimum the amount of precision machining required in its manufacture. The required electronic circuits are those which in an increasing number of laboratories are becoming idle through the introduction of automated and transistorised liquid scintillation spectrometers.

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THE APPLICATION OF PAPER CHROMATOGRAPHY TO THE STUDY OF STEROID STRUCTURE

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INTRODUCTION

Analysis of the urine from newborn infants by a paper chromatographic method[1] has revealed the presence of a large number of compounds with the properties of neutral steroids which are not prominent in the urine of adults[2].

All but a few of these occur in quantities so limited that their isolation, in pure form and in reasonable amount, for purposes of identification by all the standard macrotechniques, is extremely difficult. However, much useful information may be obtained by the direct application of chemical tests to small quantities of steroids on paper chromatograms, and the value of these tests is immeasurably increased if there is concomitant systematic application of the mathematical theory of chromatography.

MARTIN'S[3] basic theory of a set mathematical relationship between the partition coefficient of a compound and its chemical structure led to the introduction by BATE-SMITH AND WESTALL[4] of the useful term R_M, where R_M equals log $(1/R_F-1)$ and when information concerning the structure of a compound is to be derived from paper chromatographic work R_M values are employed rather than R_F values since they are more directly related to the different chemical groups contained in the molecule. In the case of the steroids, evidence that the relationships between R_M values are more nearly constant than those between R_F values can be found in the results of REINEKE[5], NEHER[6], KABASAKALIAN AND BASCH[7] and BUSH[8].

In a chromatographic system, and even in any one chromatography tank, reproducible R_F values are only achieved if there is absolute stability of a number of factors[9], and the BUSH systems, which require prolonged equilibration of solvents and paper, may present problems in the maintenance of tank equilibrium. They are also markedly influenced by changes in temperature[7]. The present analysis of the mean R_F and R_M values obtained by the repeated chromatography of standard steroids in BUSH systems under strictly controlled conditions has been undertaken in an attempt to check the accuracy of the results in chromatographic systems which are being utilized in the identification of unknown steroid compounds.

The difference between two R_M values is the ΔR_M and BUSH[8] has used the term ΔR_{Mg} for any ΔR_M value due to the substitution of another group for a hydrogen atom. He has defined ΔR_{Mr} as any ΔR_M value due to a change of molecular structure other than the simple substitution of a hydrogen atom. These terms and others advocated by BUSH[8] have been adopted for the presentation of this report.

CHROMATOGRAPHIC SYSTEMS

Bush L/85: Light petroleum (b.p. 100–120°)–methanol–water (100:85:15, by vol.). Temperature 23°. Equilibration 15 h.

Bush LB21/80: Light petroleum (b.p. 100–120°)–benzene–methanol–water (67:33:80:20, by vol.). Temperature 28°. Equilibration 5 h.

Bush T/75: Toluene–methanol–water (100:75:25, by vol.). Temperature 28°. Equilibration 5 h.

Bush LB21/A85: Light petroleum (b.p. 100–120°)–benzene–glacial acetic acid–water (67:33:85:15, by vol.). Temperature 28°. Equilibration 3 h.

In all cases, strips of Whatman No. 42 paper (50 × 2.5 cm) have been used. All paper has been washed as suggested by BUSH AND WILLOUGHBY[10].

CONTROL OF CONDITIONS

All chromatography tanks are screened from draughts in rooms with thermostatic control at 23° and 28° ± 0.5°. A constant circulation of air throughout the rooms is maintained by fans. Only a few square feet of the internal walls of the chromatography rooms are in structural contact with the external walls of the building and the areas concerned have been lined with insulating material.

The internal milieu of the tanks is satisfactorily maintained by paper lining the ends of the tanks dipping into the mobile phase, while a curtain of lint suspended from a glass rod or wire stretching along the length of the centre of the tank dips into the stationary phase contained in conical flasks in the bottom of the tank. The solvent front can thereby be seen readily on all papers in the tank. Each time tanks are loaded the presence of sufficient stationary phase is checked and the beakers are replenished only with stationary phase which has recently been prepared and equilibrated. In the case of solvent front runs a measured volume of mobile phase is added for each run. When only a few sample papers are to be run in a tank, resolution is improved by including blank strips.

The use of dyestuffs in chromatography, as recommended by NEHER, MEYSTRE AND WETTSTEIN[11] and by BUSH[8] has proved invaluable in obtaining accurate measurements of R_F values.

RESULTS

The mean R_F values for a large number of steroids and for dyes developed in the systems L/85, LB21/80, T/75 and LB21/A85, obtained from a series of runs, are given in Tables I to IV.

The R_F values are given corrected to three decimal places since, in most instances, each is the mean of a number of results. In any single run an R_F value cannot be measured to this degree of accuracy, but R_F values have not been corrected to two decimal places in the present report since the mean values obtained by arithmetic have been used in the calculation of the R_M values. ΔR_M values in these systems are presented in Tables V to X.

TABLE I

MEAN R_F AND R_M VALUES FOR STEROIDS AND A DYE IN BUSH SYSTEM L/85
Whatman No. 42 paper; Temperature 23°.

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
P[4]-11β-ol-3,20-one	11β-OH-progesterone	2	0.04	1.3802
αA-3α-ol-11,17-one	11-oxoandrosterone	1	0.04	1.3802
A[4]-3,11,17-one	adrenosterone	53	0.06	1.1965
P[4]-17α-ol-3,20-one	17α-OH-progesterone	2	0.08	1.0607
A[4]-17β-ol-3-one	testosterone	4	0.10	0.9542
A[4]-17α-ol-3-one	*cis*-testosterone	4	0.13	0.8255
αA-3β-ol-17-one	epiandrosterone	1	0.17	0.6886
A[5]-3β-ol-17-one	DHA	63	0.19	0.6269
βA-3α-ol-17-one	aetiocholanolone	62	0.245	0.4888
A[4]-3,17-one	androstenedione	12	0.31	0.3475
αA-3α-ol-17-one	androsterone	21	0.34	0.2880
P[5]-3β-ol-20-one	pregnenolone	2	0.39	0.1942
αA-3,17-one	5α-androstanedione	1	0.50	0.0
P[4]-3,20-one	progesterone	3	0.56	—0.1046
βP-3,20-one	pregnanedione	8	0.72	—0.4101
	Waxoline purple	21	0.80	—
αA-17-one	5α-androstan-17-one	2	0.87	—0.8268

TABLE II

MEAN R_F AND R_M VALUES FOR STEROIDS AND DYES IN BUSH SYSTEM LB21/80
Whatman No. 42 paper; Temperature 28°.

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
P[4]-17α,21-ol-3,11,20-one	cortisone E	4	0.005	2.3077
βP-17α,21-ol-3,11,20-one	dihydrocortisone DHE	4	0.017	1.7753
P[4]-6β,21-ol-3,20-one	6β-OH-DOC	4	0.026	1.5805
P[4]-11β,17α-ol-3,20-one	21-deoxy F	9	0.030	1.5096
P[4]-11β,21-ol-3,20-one	corticosterone B	23	0.037	1.4142
A[4]-19-ol-3,17-dione	19-OH-androstenedione	1	0.040	1.3802
βP-3α,17α-ol-11,20-one		4	0.045	1.3267
P[4]-17α,21-ol-3,20-one	compound S	7	0.056	1.2269
P[4]-21-ol-3,11,20-one	Kendall's compound A	10	0.073	1.1038
A[5]-3β-ol-7,17-one	7-oxodehydroepiandrosterone	6	0.079	1.0663
	Lacquer violet	96	0.081	—
βA-3α,11β-ol-17-one	11β-OH-aetiocholanolone	78	0.104	0.9353
P[4]-16α-ol-3,20-one	16α-OH-progesterone	2	0.108	0.9170
P[4]-17α,21-ol-3,11,20-one(-21-OAc)	cortisone-21-acetate	1	0.122	0.8572
αP-11β,21-ol-3,20-one	allodihydro B	13	0.138	0.7956
αA-3α,11β-ol-17-one	11β-OH-androsterone	75	0.161	0.7169
A[4]-11β-ol-3,17-one	11β-OH-androst-4-ene-3,17-dione	75	0.195	0.6157
βA-3α-ol-11,17-one	11-oxoaetiocholanolone	81	0.252	0.4725
βP-17α,21-ol-3,11,20-one(-21-OAc)	dihydro E 21-acetate	1	0.254	0.4679
P[4]-6β-ol-3,20-one	6β-OH-progesterone	9	0.276	0.4188
P[5]-3β,17α-ol-20-one	17α-OH-pregnenolone	1	0.292	0.3847
P[4]-11β-ol-3,20-one	11β-OH-progesterone	10	0.300	0.3680
αA-3α-ol-11,17-one	11-oxoandrosterone	79	0.308	0.3516

(*continued on p.* 365)

TABLE II (*continued*)

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
P^5-3β,21-ol-20-one	21-OH-pregnenolone	28	0.313	0.3414
βP-3α,17α-ol-20-one	17α-OH-pregnanolone	40	0.344	0.2804
P^4-11β,21-ol-3,20-one(-21-OAc)	corticosterone-21-acetate	1	0.349	0.2707
A^4-3,11,17-one	adrenosterone	92	0.395	0.1853
βP-3α,21-ol-20-one	tetrahydro DOC	5	0.411	0.1562
A^4-17β-ol-3-one	testosterone	49	0.424	0.1329
P^4-21-ol-3,11,20-one(-21-OAc)	compound A-21-acetate	1	0.425	0.1313
P^4-17α-ol-3,20-one	17α-OH-progesterone	15	0.432	0.1189
P^4-21-ol-3,20-one	DOC	25	0.478	0.0382
A^4-17α-ol-3-one	*cis*-testosterone	14	0.482	0.0315
	Neher dye F_{11}	52	0.495	—
αA-3,11,17-one	5α-androstanetrione	7	0.550	—0.0872
A^5-3β-ol-17-one	dehydroepiandrosterone	86	0.571	—0.1244
P^4-20α-ol-3-one		3	0.602	—0.1798
βA-3α-ol-17-one	aetiocholanolone	16	0.610	—0.1945
αA-3α-ol-17-one	androsterone	16	0.694	—0.3556
βP-21-ol-3,20-one	dihydro DOC	3	0.710	—0.3893
A^4-3,17-one	androst-4-ene-3,17-dione	7	0.776	—0.5391
P^5-3β-ol-20-one	pregn-5-enolone	2	0.778	—0.5452
αA-3,17-one	5α-androstanedione	8	0.828	—0.6819
P^4-3,20-one	progesterone	10	0.844	—0.7328
P^5-3β,17α-ol-20-one(-3-OAc)	17α-OH-pregnenolone-3-acetate	1	0.857	—0.7773
βA-3α-ol-11,17-one(-3-OAc)	11-oxoaetiocholanolone-3-acetate	2	0.866	—0.8097
P^4-6β-ol-3,20-one(-6-OAc)	6β-OH-progesterone-6-acetate	1	0.882	—0.8729
αA-17-one	5α-androstan-17-one	8	0.917	—1.0410
	Waxoline purple	59	0.919	—

TABLE III

MEAN R_F AND R_M VALUES FOR STEROIDS AND DYES IN BUSH SYSTEM T/75

Whatman No. 42 paper; Temperature 28°.

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
P^4-6β,11β,17α,21-ol-3,20-one	6β-OH F	4	0.002	2.6981
P^4-6β,17α,21-ol-3,11,20-one	6β-OH E	48	0.016	1.7889
P^4-11β,17α,20α,21-ol-3-one		1	0.029	1.5247
P^4-11β,17α,20β,21-ol-3-one		3	0.042	1.3581
βP-3α,11β,17α,21-ol-20-one	THF	48	0.067	1.1440
αP-3α,11β,17α,21-ol-20-one	allo-THF	9	0.089	1.0103
αP-3β,17α,21-ol-11,20-one	3β-allo-THE	3	0.096	0.9741
βP-3α,17α,21-ol-11,20-one	THE	52	0.113	0.8949
P^4-11β,17α,21-ol-3,20-one	cortisol, F	130	0.156	0.7339
	Neher dye F_{14}	10	0.207	—
P^4-11β,21-ol-18-al-3,20-one	aldosterone	3	0.232	0.5211
βP-11β,17α,21-ol-3,20-one	dihydrocortisol	8	0.283	0.4041
P^4-17α,21-ol-3,11,20-one	cortisone, E	107	0.290	0.3888
αP-3β,11β,21-ol-20-one	3β-allo-THB	3	0.364	0.2430
βP-3α,11β,21-ol-20-one	THB	8	0.410	0.1580
P^4-6β,21-ol-3,20-one	6β-OH DOC	4	0.424	0.1329
βP-17α,21-ol-3,11,20-one	dihydrocortisone	17	0.426	0.1300

(*continued on p.* 366)

TABLE III (*continued*)

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
αP-17α,21-ol-3,11,20-one	allodihydrocortisone	2	0.430	0.1225
βP-3α,17α,21-ol-20-one	THS	4	0.434	0.1152
βP-3α,17α-ol-11,20-one		2	0.532	—0.0555
A^5-3β,16α-ol-17-one	16α-OH DHA	2	0.534	—0.0590
βP-3α,21-ol-11,20-one	THA	8	0.536	—0.0625
P^4-11β,21-ol-3,20-one	corticosterone	30	0.606	—0.1864
	Lacquer violet	6	0.608	—
P^4-17α,21-ol-3,20-one	compound S	5	0.618	—0.2083
A^5-3β-ol-7,17-one	7-oxo-DHA	3	0.620	—0.2125
βA-3α,11β-ol-17-one	11β-OH-aetiocholanolone	1	0.638	—0.2464
	Neher dye F_5	11	0.669	—
P^4-21-ol-3,11,20-one	compound A	45	0.692	—0.3516
A^4-11β-ol-3,17-one	11β-OH-androstenedione	2	0.750	—0.4776
βA-3α-ol-11,17-one	11-oxoaetiocholanolone	2	0.797	—0.5935
A^4-17β-ol-3-one	testosterone	2	0.839	—0.7167
A^4-17α-ol-3-one	*cis*-testosterone	2	0.850	—0.7545
A^4-3,11,17-one	adrenosterone	2	0.869	—0.8210
	Neher dye F_{11}	4	0.869	—
A^5-3β-ol-17-one	DHA	3	0.878	—0.8570
P^4-21-ol-3,20-one	deoxycorticosterone	12	0.882	—0.8729
βA-3α-ol-17-one	aetiocholanolone	1	0.884	—0.8827
αA-3α-ol-17-one	androsterone	1	0.891	—0.9136
A^4-3,17-one	androstenedione	3	0.903	—0.9706
αA-3,17-one	5α-androstane-3,17-dione	2	0.927	—1.1024
	Waxoline purple	41	0.936	—
αA-3-one	5α-androstanone	1	0.939	—1.1871

TABLE IV

MEAN R_F AND R_M VALUES FOR STEROIDS AND DYES IN BUSH SYSTEM LB21/A85
Whatman No. 42 paper; Temperature 28°.

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
P^4-6β,17α,21-ol-3,11,20-one	6β-OH-E	1	0.001	2.9203
P^4-11β,17α,21-ol-3,20-one	F	1	0.012	1.9193
P^4-17α,21-ol-3,11,20-one	E	2	0.015	1.8263
βP-3α,11β,17α,21-ol-20-one	THF	2	0.0235	1.6185
βP-3α,17α,21-ol-11,20-one	THE	2	0.024	1.6093
βP-11β,17α,21-ol-3,20-one	dihydro F	1	0.033	1.4642
βP-17α,21-ol-3,11,20-one	dihydro E	1	0.036	1.4240
P^4-6β,21-ol-3,20-one	6β-OH-DOC	1	0.042	1.3549
P^4-17α,21-ol-3,11,20-one(-21-OAc)	E-21-acetate	2	0.051	1.2669
P^4-11β,17α-ol-3,20-one	21-deoxy F	1	0.053	1.2504
P^4-21-ol-3,11,20-one	A	2	0.055	1.2342
	Neher dye F_5	8	0.063	—
P^4-11β,21-ol-3,20-one	B	2	0.066	1.1516
αP-3β,11β,21-ol-20-one	3β-allo-THB	1	0.077	1.0799
P^4-16α-ol-3,20-one	16α-OH-progesterone	2	0.0855	1.0294
A^5-3β-ol-7,17-one	7-oxo-DHA	4	0.086	1.0257
βP-3α,11β,21-ol-20-one	THB	2	0.089	1.0111

(*continued on p.* 367)

TABLE IV (*continued*)

Compound	*Trivial name*	*No. of estimations*	R_F	R_M
	Lacquer violet	35	0.106	—
A^4-11β-ol-3,17-one	11β-OH-androstenedione	4	0.110	0.9080
P^4-21-ol-3,11,20-one(-21-OAc)	A-21-acetate	1	0.149	0.7567
P^4-6β-ol-3,20-one	6β-OH-progesterone	1	0.153	0.7432
P^4-11β,21-ol-3,20-one(-21-OAc)	B-21-acetate	1	0.156	0.7332
P^4-11β-ol-3,20-one	11β-OH-progesterone	1	0.165	0.7043
βA-3α,11β-ol-17-one	11β-OH-aetiocholanolone	11	0.168	0.6948
P^4-17α,21-ol-3,20-one(-21-OAc)	S-21-acetate	1	0.181	0.6556
A^4-3,11,17-one	adrenosterone	13	0.183	0.6497
αA-3α,11β-ol-17-one	11β-OH-androsterone	13	0.193	0.6213
βA-3α-ol-11,17-one	11-oxoaetiocholanolone	14	0.248	0.4817
P^4-21-ol-3,20-one	DOC	3	0.258	0.4588
αA-3α-ol-11,17-one	11-oxoandrosterone	9	0.265	0.4431
P^4-17α-ol-3,20-one	17α-OH-progesterone	1	0.278	0.4145
P^4-16α-ol-3,20-one(-16-OAc)	16α-OH-progesterone-16-acetate	1	0.286	0.3974
βP-3α,11β,17α,21-ol-20-one-(-3,21-OAc)	THF-3,21-diacetate	1	0.288	0.3931
P^5-3β,21-ol-20-one	21-OH-pregn-5-enolone	7	0.301	0.3659
	Neher dye F_{11}	45	0.334	—
A^4-17β-ol-3-one	testosterone	1	0.335	0.2978
A^4-17α-ol-3-one	*cis*-testosterone	1	0.345	0.2786
βP-3α,21-ol-20-one	tetrahydro DOC	1	0.393	0.1889
A^5-3β-ol-7,17-one(-3-OAc)	7-oxo-DHA-3-acetate	2	0.409	0.1599
A^4-3,17-one	androst-4-ene-3,17-dione	4	0.421	0.1383
A^5-3,17-one	androst-5-ene-3,17-dione	1	0.421	0.1383
P^4-21-ol-3,20-one(-21-OAc)	DOC-21-acetate	1	0.461	0.0679
αA-3α,11β-ol-17-one(-3-OAc)	11β-OH-androsterone-3-acetate	7	0.465	0.0611
A^5-3β-ol-17-one	DHA	8	0.469	0.0539
P^5-3β,21-ol-20-one(-21-OAc)	21-OH-pregn-5-enolone-21-acetate	2	0.495	0.0086
βA-3α-ol-17-one	aetiocholanolone	8	0.522	—0.0381
βA-3α,11β-ol-17-one(-3-OAc)	11β-OH-aetiocholanolone-3-acetate	6	0.542	—0.0731
βP-3α,11β,21-ol-20-one(-3,21-OAc)	THB-3,21-diacetate	1	0.548	—0.0835
αA-3α-ol-11,17-one(-3-OAc)	11-oxoandrosterone-3-acetate	4	0.562	—0.1085
αA-3α-ol-17-one	androsterone	7	0.581	—0.1421
P^4-3,20-one	progesterone	6	0.591	—0.1599
βA-3α-ol-11,17-one(-3-OAc)	11-oxoaetiocholanolone-3-acetate	9	0.608	—0.1904
αA-3,17-one	5α-androstane-3,17-dione	1	0.618	—0.2090
A^5-3β-ol-17-one(-3-OAc)	DHA-3-acetate	2	0.777	—0.5421
βP-3α,21-ol-20-one(-3,21-OAc)	tetrahydro-DOC-3,21-diacetate	1	0.792	—0.5800
αA-3α-ol-17-one(-3-OAc)	androsterone-3-acetate	2	0.795	—0.5884
P^5-3β,21-ol-20-one(-3,21-OAc)	21-OH-pregnenolone-3,21-diacetate	3	0.831	—0.6925
βA-3α-ol-17-one(-3-OAc)	aetiocholanolone-3-acetate	2	0.835	—0.7033
	Waxoline purple	58	0.858	—
αA-17-one	5α-androstan-17-one	1	0.870	—0.8268
	Sudan red	22	0.889	—

TABLE V

ΔR_{Mg} VALUES FOR HYDROXYLATION IN THE SYSTEM LB21/80
(Temperature 28°)

Substituent	*Root compound*	ΔR_{Mg}
6β-OH	P^4-21-ol-3,20-one	+1.54
	P^4-3,20-one	+1.15

(*continued on p.* 368)

TABLE V (*continued*)

Substituent	*Root compound*	ΔR_{Mg}
11β-OH	P^4-17α-ol-3,20-one	+1.39
	P^4-21-ol-3,20-one	+1.38
	βA-3α-ol-17-one	+1.13
	αA-3α-ol-17-one	+1.07
	A^4-3,17-one	+1.15
	P^4-3,20-one	+1.10
16α-OH	P^4-3,20-one	+1.65
17α-OH	P^4-21-ol-3,11,20-one	+1.20
	P^4-11β-ol-3,20-one	+1.14
	P^4-21-ol-3,20-one	+1.19
	P^5-3β-ol-20-one	+0.93
	P^4-3,20-one	+0.85
20α-OH	P^4-3,20-one	+0.55
20β-OH	P^4-3,20-one	+0.37
21-OH	P^4-11β-ol-3,20-one	+1.07
	P^4-17α-ol-3,20-one	+1.13
	P^5-3β-ol-20-one	+0.89
	P^4-3,20-one	+0.89

TABLE VI

ΔR_{Mg} VALUES FOR KETONE GROUPS IN THE SYSTEM LB21/80
(Temperature 28°)

Substituent	*Root compound*	ΔR_{Mg}
7-oxo	A^5-3β-ol-17-one	+1.13
11-oxo	P^4-17α,21-ol-3,20-one	+1.08
	P^4-21-ol-3,20-one	+1.07
	βA-3α-ol-17-one	+0.67
	αA-3α-ol-17-one	+0.71
	A^4-3,17-one	+0.72
	αA-3,17-one	+0.60

TABLE VII

ΔR_{Mr} VALUES FOR ACETYLATION IN THE SYSTEM LB21/80
(Temperature 25°)

Conversion	*Root compound*	ΔR_{Mr}
3α-OH ↓ 3α-OAc	βA-3α-ol-11,17-one	—1.28
3β-OH ↓ 3β-OAc	P^5-3β,17α-ol-20-one	—1.16
6β-OH ↓ 6β-OAc	P^4-6β-ol-3,20-one	—1.29

(*continued on p.* 369)

TABLE VII (*continued*)

Conversion	*Root compound*	ΔR_{Mr}
21-OH	P^4-17α,21-ol-3,11,20-one	—1.45
↓	βP-17α,21-ol-3,11,20-one	—1.31
21-OAc	P^4-11β,21-ol-3,20-one	—1.14

TABLE VIII

ΔR_{Mg} VALUES IN THE SYSTEM T/75
(Temperature 28°)

Substituent	*Root compound*	ΔR_{Mg}
6β-OH	P^4-11β,17α,21-ol-3,20-one	+1.96
	P^4-17α,21-ol-3,11,20-one	+1.40
	P^4-21-ol-3,20-one	+1.01
11β-OH	βP-3α,17α,21-ol-20-one	+1.03
	P^4-17α,21-ol-3,20-one	+0.94
	P^4-21-ol-3,20-one	+0.69
	βA-3α-ol-17-one	+0.64
	A^4-3,17-one	+0.49
16α-OH	A^5-3β-ol-17-one	+0.80
17α-OH	βP-3α,11β,21-ol-20-one	+0.99
	βP-3α,21-ol-11,20-one	+0.96
	P^4-11β,21-ol-3,20-one	+0.92
	P^4-21-ol-3,11,20-one	+0.74
	P^4-21-ol-3,20-one	+0.66
20α-OH	P^4-11β,17α,21-ol-3,20-one	+0.79
20β-OH	P^4-11β,17α,21-ol-3,20-one	+0.62
21-OH	βP-3α,17α-ol-11,20-one	+0.95
7-oxo	A^5-3β-ol-17-one	+0.64
11-oxo	βP-3α,17α,21-ol-20-one	+0.77
	P^4-17α,21-ol-3,20-one	+0.60
	P^4-21-ol-3,20-one	+0.52

TABLE IX

ΔR_{Mg} VALUES IN THE SYSTEM LB21/A85
(Temperature 28°)

Substituent	*Root compound*	ΔR_{Mg}
6β-OH	P^4-17α,21-ol-3,11,20-one	+1.09
	P^4-21-ol-3,20-one	+0.90
	P^4-3,20-one	+0.90

(*continued on p.* 370)

TABLE IX (*continued*)

Substituent	*Root compound*	ΔR_{Mg}
11β-OH	P^4-21-ol-3,20-one	+0.69
	βP-3α,21-ol-20-one	+0.82
	A^4-3,17-one	+0.77
	P^4-21-ol-3,20-one(-21-OAc)	+0.67
	P^4-3,20-one	+0.86
	βA-3α-ol-17-one	+0.73
	αA-3α-ol-17-one	+0.76
16α-OH	P^4-3,20-one	−1.19
17α-OH	P^4-11β,21-ol-3,20-one	+0.77
	P^4-21-ol-3,11,20-one	+0.69
	βP-3α,11β,21-ol-20-one	+0.61
	P^4-11β-ol-3,20-one	+0.55
	P^4-21-ol-3,20-one(-21-OAc)	+0.59
	P^4-3,20-one	+0.57
21-OH	P^4-11β,17α-ol-3,20-one	+0.67
	P^4-6β-ol-3,20-one	+0.61
	P^4-3,20-one	+0.62
7-oxo	A^5-3β-ol-17-one	+0.97
11-oxo	P^4-21-ol-3,20-one	+0.78
	A^4-3,17-one	+0.51
	βA-3α-ol-17-one	+0.52
	αA-3α-ol-17-one	+0.59
	αA-17-one	+0.62

TABLE X

ΔR_{Mf} VALUES IN SYSTEMS LB21/80, LB21/A85, L/85 AND T/75
(Temperature 28°)

Conversion	*Root compound*	ΔR_{Mf} *in systems*			
		LB21/80	*LB21/A85*	*L/85*	*T/75*
5β(H)-3α-OH ↓ Δ^5-3β-OH	βP-3α,17α-ol-20-one	+0.1	—	—	—
	βA-3α-ol-17-one	+0.07	+0.09	+0.14	+0.03
5β(H)-3α-OH ↓ Δ^4-3-oxo	βP-3α,11β,17α,21-ol-20-one	—	+0.30	—	−0.41
	βP-3α,17α,21-ol-11,20-one	—	+0.22	—	−0.50
	βP-3α,11β,21-ol-20-one	—	+0.14	—	−0.34
	βP-3α,17α,21-ol-20-one	—	—	—	−0.32
	βP-3α,21-ol-11,20-one	—	—	—	−0.29
5β(H)-3α-OH ↓ 5α(H)-3β-OH	βP-3α,17α,21-ol-11,20-one	—	—	—	+0.080
	βP-3α,11β,21-ol-20-one	—	+0.07	—	+0.085
	βA-3α-ol-17-one	—	—	+0.20	—
5β(H)-3α-OH ↓ 5α(H)-3α-OH	βP-3α,11β,17α,21-ol-20-one	—	—	—	−0.13
	βA-3α,11β-ol-17-one	−0.22	−0.07	—	—
	βA-3α-ol-11,17-one	−0.12	−0.04	—	—
	βA-3α-ol-17-one	−0.16	−0.10	−0.20	−0.03

DISCUSSION

R_F values of compounds have been obtained either from their position relative to that of the solvent front or from overruns in which standard compounds or dyes with known R_F values have been included. For example, in the LB21/80 system, the R_F values for adrenosterone, DOC, F_{11} and less polar compounds have been measured from runs in which the position of the solvent front has been ascertained. The R_F values of the more polar steroids have been obtained from overruns and based upon the R_F values of one or more of these reference compounds used as a marker. In the T/75 system, the R_F values of THB and compounds less polar have been calculated from runs in which the position of the solvent front has been located, while the R_F values of the more polar compounds have been calculated from overruns and based upon previously determined R_F values—that of dihydrocortisone for chromatograms which have been overrun for a limited period, and that of cortisol for chromatograms which have had prolonged overrunning.

A stable environment for chromatography is reflected in the ΔR_M values listed in Tables V to X. In all the systems it can be seen that one specific structural change in the molecule produces a change in the R_M value of a steroid compound which is very nearly constant.

In all cases, the steroids have been listed in the tables in descending order of polarity and this reveals that the ΔR_{Mg} values for oxy-groups tend to decrease as the polarity of the root compound decreases. However, the values obtained show that the ΔR_{Mg} value for a specific oxy-group is virtually constant for root substances of near polarity.

Apart from the isolated values given in previous reports from this laboratory[1,2], only a few R_F values for steroids run in these aqueous methanol systems have been published but several of the ΔR_M values obtained in the present study parallel those published by BUSH[8] for steroids run in closely related systems.

The results given here not only provide further evidence for the validity of the theory of BATE-SMITH AND WESTALL[4] but also show that chromatography is being carried out under suitably stable conditions. However, in practice, even with the strict attention to detail during chromatography which has been described, variations in R_F values do occur from one run to another. Of the systems studied, the LB21/80 and the L/85 are the most prone to show this and the possibility of errors arises in ΔR_M calculations based on the average R_F values obtained from a number of runs although the chances of serious errors are much less than in calculations based on the results from isolated runs.

In consequence, when unknown steroid compounds are submitted to chromatography during their structural investigation it is advisable to carry out a preliminary check of the agreement of the mobility of a suitable dyestuff relative to the solvent front with the average R_F value of that dye established over a period of time in the laboratory. Thereafter, unknown compounds and their derivatives may be run in the tank but it is essential to run them in association with appropriate standards and dyes. If the relationships between the R_M values of the standard compounds included in any such run are found to be consistent with those regularly achieved in the laboratory it is reasonable to assume that the average ΔR_M values established for the particular chromatographic system are applicable in considerations of the possible nature of the steroids under investigation.

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SUMMARY

Mean R_F and R_M values have been obtained for a number of steroids and dyes in BUSH aqueous methanol systems. Scrutiny of the ΔR_M values verifies the stability of the conditions achieved.

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A PAPER CHROMATOGRAPHIC TECHNIQUE FOR SCREENING VOLATILE CHEMICALS FOR THEIR REACTIVITY WITH THE CONSTITUENTS OF FOODS

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Fumigants, a class of volatile chemicals which are basically toxic to insects and mammals, have been used for the control of insects in food and other commodities throughout the world[1]. The use of such fumigants in the control of microflora on food-grains has also been reported[2-5]. One of the most important factors governing the choice or rejection of any particular chemical as a fumigant is the extent of its chemical reactivity with constituents of the food or commodity being subjected to treatment.

Some fumigants are not only unstable, and decompose when sorbed by a commodity, but also react chemically to form new compounds, which can be either degradation or addition products with the constituents in the fumigated materials. These products may be toxic and remain in the commodity as residues[4-7]. Under the Food and Drug Laws of various countries, the amount of residue which can be permitted in different foodstuffs has been prescribed. In order to establish the tolerances for residues, data on the fate of the fumigant reaction products have to be collected. The methods or techniques which have been employed for such studies are extremely time consuming and difficult[6-8].

WINTERINGHAM *et al.*[8] found that when methyl bromide is used as a fumigant a portion is physically adsorbed which irreversibly combines with one or more constituents of wheat. The gluten or protein fraction of the wheat accounted for 80% decomposition. These studies involved combined radioactive tracer and chromatographic techniques.

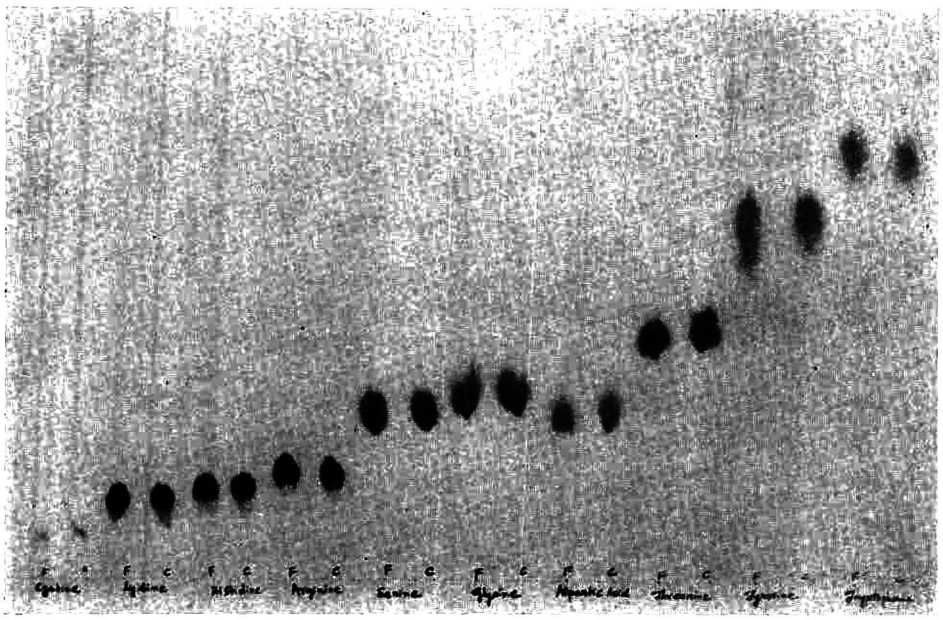
A high level of bromide residues was estimated in this laboratory[9,10] after the fumigation of cashewnuts, groundnuts and copra (dry coconut cups). Estimation of the amino acids of the fumigated and unfumigated defatted cashewnut, groundnut and coconut meals, by the paper chromatographic and colorimetric methods, showed a decrease in the concentration of methionine after fumigation[10].

In view of the above evidence of the reaction of a fumigant with amino acids it was of interest to examine paper chromatograms spotted with non-radioactive amino acids for reactivity with the gaseous chemicals during fumigation.

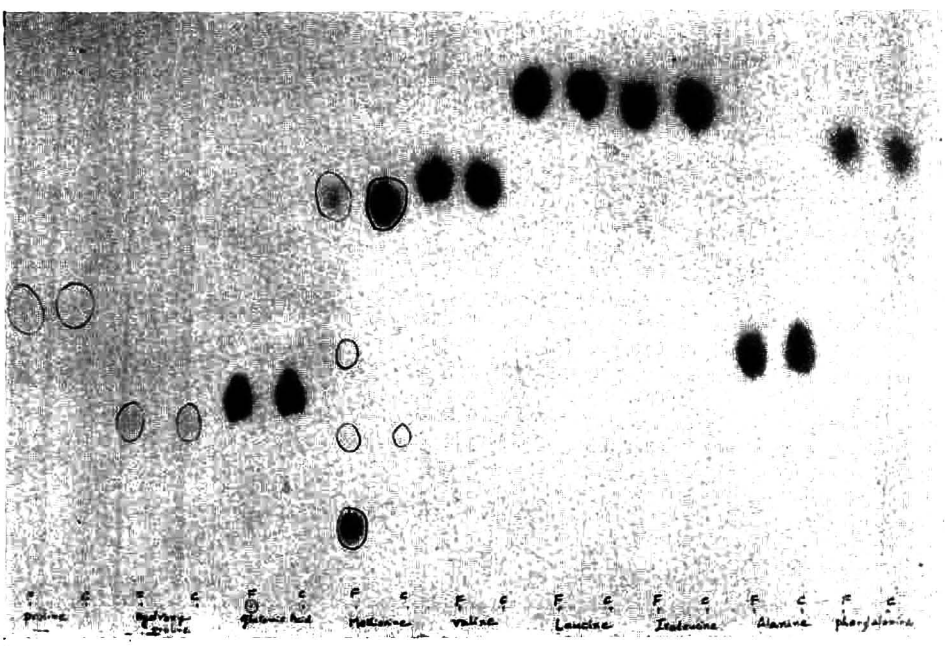
EXPERIMENTAL

Selection of an amino acid reactive with some fumigants

Ten gamma quantities of amino acids (aqueous solutions) were spotted on Whatman No. 1 filter paper (46 × 57 cm) leaving space for spotting their duplicates



(a)



(b)

Fig. 1. Reactivity of amino acids exposed for 100 h to methyl bromide in a concentration of 100 mg/l. F = fumigated; C = control. (a) From left to right: cystine, lysine, histidine, arginine, serine, glycine, aspartic acid, threonine, tyrosine, tryptophan; (b) From left to right: proline, hydroxyproline, glutamic acid, methionine, valine, leucine, isoleucine, alanine, phenylalanine.

TABLE I

REACTIVITY OF AMINO ACIDS WITH SOME FUMIGANTS: MB, EDB, MB + EDB AND EDCT* AT 100 mg/l, 25° FOR 100 h EXPOSURE

Sl. No.	*Amino acid*	*R_F values of the amino acids and additional spots if any after treatment with:*				
		MB	*EDB*	*EDB + MB*	*EDCT*	*Control*
1	Alanine	0.42	0.42	0.42	0.42	0.42
	Additional spots	Nil	Nil	Nil	Nil	Nil
2	Arginine	0.19	0.19	0.19	0.19	0.19
	Additional spots	Nil	Nil	Nil	Nil	Nil
3	Aspartic acid	0.27	0.27	0.27	0.27	0.27
	Additional spots	Nil	Nil	Nil	Nil	Nil
4	Cystine	0.09	0.09	0.09	0.09	0.09
	Additional spots	Nil	Nil	Nil	Nil	Nil
5	Glutamic acid	0.36	0.36	0.36	0.36	0.36
	Additional spots	Nil	Nil	Nil	Nil	Nil
6	Glycine	0.29	0.29	0.29	0.29	0.29
	Additional spots	Nil	Nil	Nil	Nil	Nil
7	Histidine	0.17	0.17	0.17	0.17	0.17
	Additional spots	Nil	Nil	Nil	Nil	Nil
8	Hydroxyproline	0.32	0.32	0.32	0.32	0.32
	Additional spots	Nil	Nil	Nil	Nil	Nil
9	Isoleucine	0.71	0.71	0.71	0.71	0.71
	Additional spots	Nil	Nil	Nil	Nil	Nil
10	Leucine	0.72	0.72	0.72	0.72	0.72
	Additional spots	Nil	Nil	Nil	Nil	Nil
11	Lysine	0.16	0.16	0.16	0.16	0.16
	Additional spots	Nil	Nil	Nil	Nil	Nil
12	Methionine	0.58	0.58	0.58	0.58	0.58
	Additional spots: 1	0.10	Nil	0.10	Nil	Nil
	2	0.22	0.22	0.22	0.22	0.22
	3	0.40	Nil	Nil	Nil	Nil
13	Phenylalanine	0.69	0.69	0.69	0.69	0.69
	Additional spots	Nil	Nil	Nil	Nil	Nil
14	Proline	0.47	0.47	0.47	0.47	0.47
	Additional spots	Nil	Nil	Nil	Nil	Nil
15	Serine	0.28	0.28	0.28	0.28	0.28
	Additional spots	Nil	Nil	Nil	Nil	Nil
16	Threonine	0.35	0.35	0.35	0.35	0.35
	Additional spots	Nil	Nil	Nil	Nil	Nil
17	Tryptophan	0.60	0.60	0.60	0.60	0.60
	Additional spots	Nil	Nil	Nil	Nil	Nil
18	Tyrosine	0.51	0.51	0.51	0.51	0.51
	Additional spots	Nil	Nil	Nil	Nil	Nil
19	Valine	0.61	0.61	0.61	0.61	0.61
	Additional spots	Nil	Nil	Nil	Nil	Nil

* MB = methyl bromide; EDB = ethylene dibromide; MB + EDB = a mixture of methyl bromide and ethylene dibromide; EDCT = ethylene dichloride–carbon tetrachloride.

after the fumigation of the spots. These spotted papers were subjected to the action of vapours of the fumigants at 25 ± 1° in a concentration of 100 mg/l in glass tubes for 100 h by the procedure described below.

The spotted papers were placed inside glass tubes (150 cm length, 5 cm diameter) fitted with glass stop cocks, terminating on the inside in small extraction thimbles which acted as evaporators for the liquid fumigants. To admit the fumigant, the tube

TABLE II

EFFECT OF DIFFERENT FUMIGANTS ON METHIONINE

Sl. No.	*Fumigants*	*Chemical formulae*	*No. of additional spots*	*R_F values*				*% Recovery of methionine*	*Extent of reaction (%)*
				1	*2*	*3*	*4*		
1	Acetone	CH_3COCH_3	—	—	0.22	—	—	95	5
2	Acrylonitrile	CH_2CHCN	—	—	0.22	—	—	98	2
3	Ammonia	NH_3	—	—	0.22	—	—	98	2
4	Aniline	$C_6H_5NH_2$	—	—	0.22	—	—	98	2
5	Benzene	C_6H_6	—	—	0.22	—	—	95	5
6	β-Propiolactone	$CH_2CH_2C{=}O$ (O)	2	0.09	0.22	—	0.89	62	38
7	Carbon disulphide	CS_2	—	—	0.22	—	—	98	2
8	Carbon tetrachloride	CCl_4	—	—	0.22	—	—	93	7
9	Chlorobenzene	C_6H_5Cl	—	—	0.22	—	—	89	11
10	Chloroform	$CHCl_3$	—	—	0.22	—	—	98	2
11	Chloropicrin	CCl_3NO_2	—	—	0.22	—	—	88	12
12	Ethyl acetate	$CH_3COOC_2H_5$	—	—	0.22	—	—	98	2
13	Ethyl acetoacetate	$CH_3COCH_2COOC_2H_5$	—	—	0.22	—	—	98	2
14	Ethyl bromide	C_2H_5Br	1	0.10	0.22	—	—	81	19
15	Ethyl formate	$HCOOC_2H_5$	—	—	0.22	—	—	98	2
16	Ethylene chlorhydrin	CH_2ClCH_2OH	—	—	0.22	—	—	98	2
17	Ethylene dibromide	$C_2H_4Br_2$	—	—	0.22	—	—	93	7
18	Ethylene dichloride	$C_2H_4Cl_2$	—	—	0.22	—	—	95	5
19	Ethylene dichloride–carbon tetrachloride (EDCT) (3:1, v/v)		—	—	0.22	—	—	93	7
20	Ethyl methyl ketone	$CH_3COC_2H_5$	—	—	0.22	—	—	95	5
21	Lindane (γ-BHC)	$C_6H_6Cl_6$	—	—	0.22	—	—	98	2
22	Menthol	$CH_3(C_3H_7)C_6H_9{-}OH$	—	—	0.22	—	—	93	7
23	Methyl alcohol	CH_3OH	—	—	0.22	—	—	98	2
24	Methyl bromide	CH_3Br	2	0.10	0.22	0.40	—	48	52
25	Methyl bromide + ethylene dibromide (EDB + MB) (1:1, w/w)	$CH_3Br + C_2H_4Br_2$	1	0.10	0.22	—	—	71	29
26	Methyl cyanide	CH_3CN	—	—	0.22	—	—	98	2
27	Methyl iodide	CH_3I	1	0.11	0.22	—	—	88	12
28	Hexane (normal)	$CH_3(CH_2)_4CH_3$	—	—	0.22	—	—	96	4
29	Naphthalene	$C_{10}H_8$	—	—	0.22	—	—	93	7
30	Nitrobenzene	$C_6H_5NO_2$	—	—	0.22	—	—	88	12
31	Nitromethane	CH_3NO_2	—	—	0.22	—	—	95	5
32	p-Dichlorobenzene	$C_6H_4Cl_2$	—	—	0.22	—	—	98	2
33	Phosphine	PH_3	—	—	0.22	—	—	97	3
34	Pyridine	C_5H_5N	—	—	0.22	—	—	98	2
35	Quinoline	$C_6H_4N{=}CHCH{=}CH$	—	—	0.22	—	—	94	6
36	Thymol	$CH_3(C_3H_7)C_6H_3OH$	—	—	0.22	—	—	93	7
37	Trichloroethylene	$CHCl{=}CCl_2$	—	—	0.22	—	—	98	2
38	Vapona (DDVP), O,O-dimethyl-2,2-dichlorovinyl phosphate	CH_3O, CH_3O, $P{=}O$, $OCHCCl_2$	1	0.10	0.22	—	—	83	17
39	Xylene	$C_6H_4(CH_3)_2$	—	—	0.22	—	—	89	11
40	Control (unfumigated)	—	—	—	0.22	—	—	98	2

was evacuated to a pressure of 2 in. absolute and the liquid fumigants were pipetted into the chambers by connecting the pipette to the stop-cock and opening the stop-cock to draw in the fumigant, at the same time restoring the atmospheric pressure. While administering methyl bromide essentially the same technique was adopted, but the fumigant was administered as a gas from a calibrated 2-way "Strand" type flask filled to atmospheric pressure with methyl bromide and displaced into the fumigation chamber by running calcium chloride saturated water into the flask[11]. When ethylene dibromide–methyl bromide mixture was used the micro-pipette and the "strand" flask were connected in series while dosing, the latter being disconnected while restoring atmospheric pressure[11].

In the experiment on the selection of a reactive amino acid for further studies only four commonly used fumigants were used. These were ethylene dibromide, methyl bromide, a mixture of ethylene dibromide and methyl bromide and a mixture of ethylene dichloride and carbon tetrachloride. After exposure to the fumigants and aeration of vapours and spotting the corresponding unfumigated amino acids alongside the treated ones, the chromatograms were irrigated with a butanol–acetic acid–water (4:1:5) solvent system by the descending chromatographic technique.[12] The spots were developed and examined for their R_F values, comparison being made with the corresponding unfumigated amino acids. On developing the paper chromatogram it was observed that out of the nineteen amino acids tested, additional spots were only observed on the paper chromatogram in the fumigated samples in the case of methionine (see Table I). Out of the fumigants tried in the experiment, indication of the presence of reaction products of methionine was most obvious in the chromatograms which were exposed to methyl bromide (see also Fig. 1). This led to the possibility of developing a new and rapid screening technique for the chemical reactivity or inertness of the fumigants with the constituents of foods.

Screening for reactivity of fumigants with methionine

A large number of chemicals (Table II) which are either conventional fumigants or have some prospect of being used as fumigants for disinfestation of foods were screened for their reactivity by exposing the chromatographic papers (28 × 8 cm) spotted with methionine (5 to 10 γ) in aluminium alloy pressure cookers (4.75 l) fitted with glass stop-cocks, terminating on the inside into small extraction thimbles which acted as evaporators for the liquid fumigants (see Fig. 2). The procedure for the application of the liquid and gaseous fumigants was the same as that followed in the previous experiment. In case of solids, weighed quantities of the crystals were placed inside the chamber and the lids were immediately closed. For the generation of phosphine the commercial preparation "Phostoxin" tablet (M/s Degesch) was used. All the chemicals were applied to give a nominal concentration of 100 mg/l, except in the case of β-propiolactone, which was applied at a concentration of 50 mg/l.

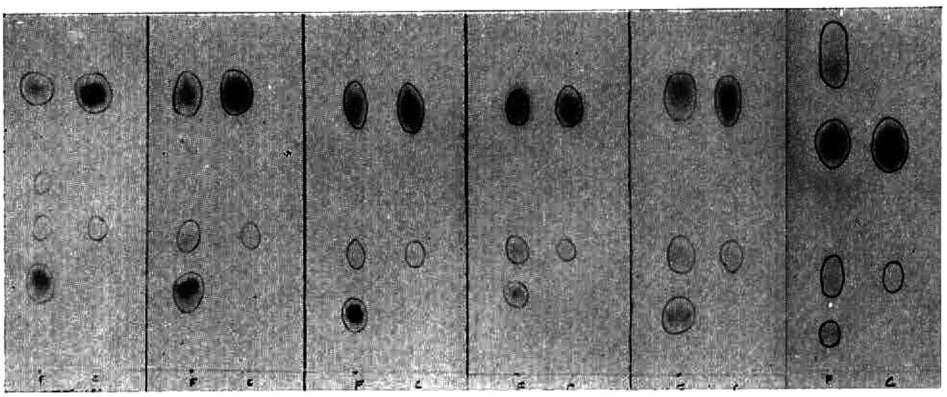
Unreacted methionine on the chromatograms was estimated by eluting the ninhydrin colour of the spots in 75% alcohol and measuring the intensities in a Klett Summerson colorimeter[13]. R_F values of new reaction products resulting from the action of the fumigant with methionine and the extent of reaction after 100 h of exposure to a 100 mg/l concentration at 25 ± 1° were noted.

Fig. 2. Fumigation assembly consisting of: (1) fumigation chamber; (2) fumigant inlet; (3) amino acid spotted paper roll suspended from centre; (4) methyl bromide container; (5) ethylene dibromide; (6) micropipette for liquids; (7) "strand" type gas applicator.

RESULTS AND DISCUSSION

Of the nineteen amino acids examined, methionine was found to be the most reactive amino acid with the fumigants used for screening (see Table I and Fig. 1). It was also noted that even the unfumigated spot of methionine gave rise to an additional spot on the paper chromatogram, which was present in all the experimental chromatograms. This common additional spot had an R_F value of 0.22 in all the chromatograms. Results in Table II revealed that depending on the reactivity of the fumigant with methionine, additional spots were present.

Fumigation with methyl bromide and β-propiolactone resulted in two spots of reacted products of methionine in addition to the common spots at R_F value of 0.58 (unreacted methionine) and R_F 0.22 (oxidised methionine). Vapona, ethyl bromide and methyl iodide reacted with the methionine spot and resulted in one additional spot indicating their reactivity with methionine even at room temperature (see Fig. 3a). The rest of the fumigants did not show discernible new spots of reaction products. Unreacted methionine was estimated from all chromatograms. The recovery data and the intensity of the spots indicated that chloropicrin, nitrobenzene, chlorobenzene, xylene, ethylene dichloride-carbon tetrachloride also reacted slightly with methionine. They also resulted in an increase in intensity of the common additional spot of R_F value 0.22 (see Fig. 3b).



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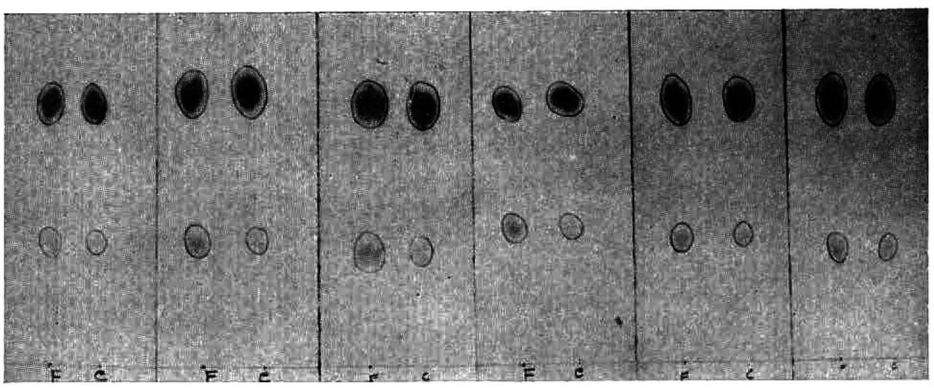

(b)

Fig. 3. Effect of some fumigants on methionine. F = fumigated; C = control. (a) From left to right: methyl bromide, methyl bromide + ethylene dibromide, methyl iodide, ethyl bromide, vapona, β-propiolactone; (b) From left to right: chlorobenzene, nitrobenzene, chloropicrin, xylene, carbon tetrachloride, ethylene dichloride.

Since the additional spots which were ninhydrin positive only could be seen on the paper chromatogram, it is possible that the other reaction products of methionine, those which are ninhydrin negative, could not be detected by the procedure. The data on the recovery of methionine and also the extent of reactivity as presented in Table II give some indication about the total reaction brought about by the fumigant with the methionine spots. Further investigations will therefore be needed to obtain data on the different reaction products which are ninhydrin positive as well as those which are negative. However, the recovery data of the unreacted methionine from the chromatogram, after fumigation of the spots, could indicate the extent of reaction. Scanning the intensity and area of the spots would give direct measures of the extent of losses due to the reactivity of the fumigants.

Results presented in Tables I and II and Figs. 1 and 3 indicated that the reactivity with methionine could be obtained with extreme rapidity and reliability for fumigants for which no information is previously available. Out of the volatile

chemicals screened for their reactivity with methionine, the following showed a high degree of reactivity: methyl bromide, methyl iodide, ethyl bromide, β-propiolactone and vapona (Fig. 3a).

This new *in situ* reaction of chemical constituents of foods on the spotted chromatographic paper with the chemical vapours screened for use as fumigants for insect control in foodstuffs, seems to offer a simple, reliable and extremely sensitive technique for investigating the possibility of their safe use. As non-reactivity with the component of food is a very desirable property for a chemical to be applied in fumigation of foodstuffs, the candidate chemicals can be selected on the basis of the direct evidence as obtained by this new technique. The essential constituents such as proteins, sugars, vitamins, fats and minerals should not be reacted upon by the fumigants so as to render them either unavailable as nutrients or make them toxic. Hitherto the techniques which are employed for the reactivity of fumigants are indirect and extremely laborious. Due to this fact, very little is known even about the fate and nature of residues of even the commonly used fumigants. With this technique the newly introduced fumigants such as vapona (DDVP), methyl iodide, β-propiolactone and many others could be studied for their reactivity with methionine even in microquantities of as low as 5 γ.

The chemical constituents of foods which can be detected and estimated by a paper chromatographic technique can be subjected to the action of fumigant vapour and examined for their reactivity by the technique described in this communication. This seems to have opened a vast possibility for rapid screening of candidate fumigants for their safety for use on foods. Many plant products, drugs, and foods require disinfestation treatment for safe storage but before they are subjected to the action of these volatile chemicals, quick and reliable information about their non-reactivity with any particular constituents for which the materials are valued should be obtained. In such cases this type of technique seems to offer great scope.

Similar studies using this technique are in progress with vitamins, sugars and fatty acids, for their susceptibility to reaction with fumigants.

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SUMMARY

A new technique is reported on the rapid screening of fumigants for their reactivity or non-reactivity with chemical constituents of foods particularly those which can be studied by paper chromatography. In the present study, extremely low quantities of amino acids spotted on paper and exposed to the volatile chemicals indicated high reactivity of methyl bromide, methyl iodide, ethyl bromide, vapona and β-propiolactone with methionine, after development of the chromatogram. This chromatographic technique offers good scope for picking out the non-reactive fumigants with ease, rapidity and reliability. It can be used as a tool for predicting the

reactivity of the chemical with the constituents of the food, and the residues which a fumigant would leave in fumigated foodstuffs during exposure.

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THE PAPER PARTITION CHROMATOGRAPHY OF UNSATURATED LIPIDS AS THEIR π-COMPLEXES WITH SILVER IONS

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The ability of olefin π-bonds to form unstable co-ordination complexes with silver ions has been known for many years[1]. More recently these π-complexes have been widely used for the separation of unsaturated lipids by adsorption chromatography[2,3]. We decided to study the application of π-complexes to the reversed-phase partition chromatography of lipids since no reports on this subject have been published so far.

EXPERIMENTAL

The general chromatographic procedure employed has been described earlier[4–6]. Chromatographic paper strips (27 × 4.5 cm) were impregnated with a 2 % solution of the stationary non-polar phase (dodecane). Fatty acid methyl esters (500–5000 μg) prepared by methanolysis of linseed, cottonseed, mustard and parsley oils[7] were applied along a baseline on the paper strips. The chromatograms were developed for 12–48 h with 70–90 % aqueous methanol saturated with dodecane and silver nitrate at room temperature, washed with 5 % aqueous nitric acid and water, and kept in the hot air current for 5–10 min[7] to remove dodecane. The lipid spots were visualized by staining with Sudan black B, and eluted with *n*-hexane. The methyl esters free from dodecane were identified by gas–liquid chromatography[6]. The methyl ester retention volume relative to methyl myristate, the number of carbon atoms and double bonds and the position of double bonds in the aliphatic chain were determined as described earlier[8]. The structure of fatty acids was referred to by shorthand designation[9].

The cottonseed oil triglycerides were separated into fractions of different polarity[6], hydrocarbons, b.p. 230–260°, being the stationary phase. Single fractions[4] were then separated in a 97.5–100 % methanol–$AgNO_3$/dodecane solvent system for 24–36 h[5]. Individual triglycerides were converted into fatty acid methyl esters[10]. The latter were determined by gas–liquid chromatography[6].

RESULTS AND DISCUSSION

The R_2 values (R_F ratio of a given substance and of butyl hexabromostearate[4]) and relative retention volumes of saturated and unsaturated fatty acid methyl esters are shown in Table I. The acids were identified using a linear relationship between the carbon number in the aliphatic chain and the $\log_{10}$ (relative retention volume) for a homologous series (Fig. 1).

One can conclude from these results that "critical pairs" of saturated and unsaturated esters having the same polarity constant $K_2 = 100 - m + 2e$, where $m =$ carbon number and $e =$ double bond number[4,5], can be separated in the silver nitrate-containing solvent system. The same effect was achieved by quantitative double bond bromination of lipids[5], but in the present solvent system the saturated lipids always remain on a baseline of the chromatogram.

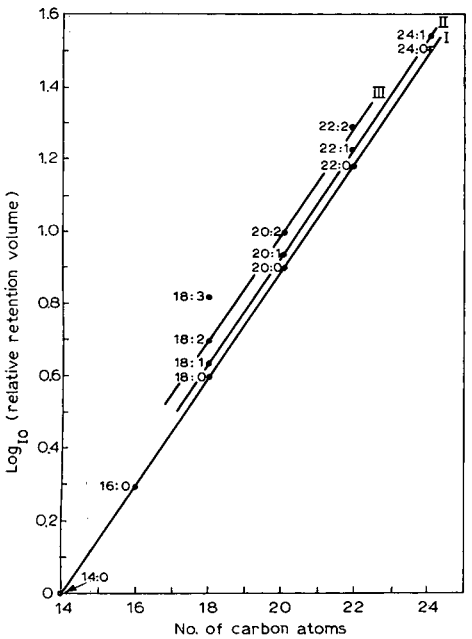

Fig. 1. The relationship between the logarithm of the relative retention volume of fatty acid methyl esters and the number of carbon atoms and double bonds in the aliphatic chain. (I) Saturated fatty acids; (II) monounsaturated fatty acids; (III) diunsaturated fatty acids.

The relative mobility of complexes is directly proportional to the number of double bonds and inversely proportional to carbon atoms number (Table I); therefore, the separation in a silver nitrate system may be regarded as the true partition chromatography[7]. Nevertheless unsaturated esters behave differently in the reversed-phase systems of different composition. Unsaturated esters having the same K_2 value do not separate in a system which does not contain complex-forming agents[11]. In a silver nitrate partition system such "critical pairs" (20:1[11] and 22:2[13,16], 18:1[9] and 20:2[11,14]) separate readily (Table I).

The chromatographic mobility of esters depends also upon the number of methylene groups between the double bonds. For example, the great difference between the R_2 values of 18:1[9] and 18:2[9,12], or of 18:2[9,12] and 18:3[9,12,15] (Table I) may result because methyl linoleate or methyl linolenate, besides having an additional double bond, have two of their double bonds widely separated and are in a more favourable position for π-complex formation[12]. Unsaturated esters are also separated according to the position of the double bonds in the chain. So methyl oleate (18:1[9]) may be completely separated from methyl petroselinate (18:1[6]) (Table I).

J. Chromatog., 17 (1965) 382–386

TABLE I

RELATIVE MOBILITY OF FATTY ACID METHYL ESTERS IN PARTITION AND GAS–LIQUID CHROMATOGRAPHIC SYSTEMS

Methyl esters	*16:0*	*18:0*	*18:1*^{9}	*18:1*^{6}	*18:2*^{9,12}	*18:3*^{9,12,15}	*20:0*	*20:1*^{11}	*20:2*^{11,14}	*22:0*	*22:1*^{13}	*22:2*^{13,16}	*24:0*	*24:1*^{15}
R_2	0	0	1.01	1.29	4.66	6.24	0	0.85	1.66	0	0.32	1.41	0	0.22
Relative retention volume	1.99	3.89	4.31	4.33	5.23	6.66	7.75	8.31	9.96	14.72	16.02	21.32	28.26	30.72

TABLE II

SEPARATION AND IDENTIFICATION OF TRIGLYCERIDES

K_2	R_2	K_3	R_2'	*Fatty acid composition (mole %)**												*Triglyceride identification*
				14:0		*16:0*		*16:1*^{9}		*18:0*		*18:1*^{9}		*18:2*^{9,12}		
				f	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	
		51	0.34	1.0	—	65.7	66.7	—	—	—	—	—	—	33.3	33.3	*PLL***
		54	0.50	0.2	—	33.6	33.3	—	—	—	—	33.7	33.3	32.5	33.3	*POL*
51	0.21	56	0.62	0.9	—	0.5	—	0.6	—	32.4	33.3	—	—	65.5	66.7	*SLL*
		57	0.70	—	—	1.8	—	0.9	—	0.5	—	65.0	66.7	31.8	33.3	*OOL*
		54	0.70	0.7	—	32.4	33.3	—	—	—	—	1.3	—	65.6	66.7	*PLL*
53	0.25	57	0.89	0.3	—	0.7	—	0.6	—	—	—	32.8	33.3	65.6	66.7	*OLL*
55	0.32	57	1.02	0.3	—	—	—	—	—	—	—	0.5	—	99.2	100	*LLL*

* f = found fatty acid composition; c = composition calculated for identified triglycerides.
** P, S, O and L = acyls of palmitic, stearic, oleic and linoleic acids.

The separation of lipids on silver nitrate-impregnated silicic acid depends only upon differences in the degree of unsaturation[13,14,15], the position of double bonds in chain[16], and the geometric configuration of the double bonds[2,17]; the aliphatic chain length has no effect on the adsorption behaviour of lipids. In the present method the R_2 value depends both on the number and position of double bonds and on the chain length of unsaturated lipids. Therefore, this method combines the advantages of Ag^+–silicic acid chromatography and ordinary reversed-phase partition chromatography.

More than 10 mg of methyl esters can be separated on the standard strip (27 × 4.5 cm); so the present procedure may be used on a preparative scale. The gas-chromatographic data show that eluted esters are more than 99 % pure.

The results of the separation of cottonseed oil triglycerides are shown in Table II. The properties of unsaturated triglyceride co-ordination complexes were characterized by equation $K_3 = m - s$, where K_3 = polarity constant of triglycerides with substituted double bonds and s = number of saturated acyls in a triglyceride molecule[4]. As shown in Table II, the original non-substituted glycerides with the same polarity constant K_2 have the same R_2 value and form a separate chromatographic zone[4,5].

In the silver nitrate-containing reversed-phase system a new polarity gradient depending upon differences in the number of carbon atoms and saturated acyls in a triglyceride is established. Each new polarity constant K_3 corresponds to its own R_2' coefficient value. It follows that single zones obtained by partition chromatography of π-complexes are individual triglycerides. The fatty acid composition and identification of separated glycerides are given in Table II. There is a satisfactory agreement between calculated and found values.

By comparison of the data of Table II with those obtained on separation of brominated triglycerides[4–6] it may be concluded that there is a marked analogy in the chromatographic behaviour of bromides and π-complexes of triglycerides. This conclusion seems to disagree with findings of DUTTON *et al.*[12], who studied the separation of unsaturated lipids by countercurrent distribution in the system hexane/0.2 *M* silver nitrate in 90 % methanol. These workers claim that differences in partition coefficients of substances being fractionated depend upon the tendency of the substances to complex with the silver ion and remain in the lower polar phase. No silver ion is present in the upper non-polar phase where only free unsubstituted lipids are contained. Our results demonstrate that separation of unsaturated lipids in the silver nitrate-containing reversed-phase system depends upon polarity differences of π-complexes. These complexes, like the brominated glycerides, are partitioned continuously between polar and non-polar phases of the chromatographic system. The advantage of the present method over the method of bromination lies in the possibility of analytical and preparative isolation of native individual triglycerides from natural sources.

Thin-layer chromatography on silicic acid impregnated with silver nitrate makes possible the separation of triglycerides into classes according to the number of double bonds[14,15]. The same separation may be obtained by the present method; moreover, our system permits the separation of the triglycerides with the same degree of unsaturation and same polarity constant but differing by the saturated fatty acid acyl content, *e.g.*, dioleolinolein and stearodilinolein (Table II).

The results obtained suggest that the proposed separation method of complex lipid mixtures may be used on an analytical and preparative scale, particularly in conjunction with partition, adsorption, and gas–liquid chromatography, as well as other modern methods of lipid analysis.

SUMMARY

Unsaturated fatty acid methyl esters and triglycerides were separated as their silver ion π-complexes by reversed-phase partition chromatography in the system 70–100 % aqueous methanol, saturated with silver nitrate and dodecane/dodecane. The fatty acid composition of single fractions was determined by gas–liquid chromatography. The unsaturated methyl esters were completely separated from saturated ones of the same polarity, and from unsaturated esters with different carbon atom or double bond number and different position of the double bonds in the aliphatic chain. Triglyceride fractions of the same polarity were fractionated into individual compounds according to double bond or saturated fatty acyls number. The method may be used on an analytical or preparative scale.

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CHROMATOGRAPHY ON ION EXCHANGE PAPERS

XIV. SOME GENERAL REMARKS ON FRONTAL ANALYSIS PHENOMENA DURING DEVELOPMENT

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INTRODUCTION

It has been shown repeatedly that ion exchange papers may yield the same information as batch equilibration or column experiments and that D values can be calculated from R_F values by the simple relation:

$$D = \left(\frac{1}{R_F} - 1\right) \frac{A_L}{A_S}$$

The values so obtained are (within the accuracy of the measurements) identical to those obtained by other methods. A comparison has also been reported between instability constants measured with ion exchange papers and those by other methods with very satisfactory results[1].

There are however differences between ion exchange papers and column experiments which are due to differences in technique and not to differences in principle, and work had been reported which claimed that no analogy can be drawn between the two techniques[2].

It is well known that oxidation reactions may take place on paper strips which cannot occur inside columns. Most workers can also detect at sight when a substance, after being spotted on the paper and dried, crystallises out instead of remaining "in solution". A further factor whose effect was known to us and automatically accounted for, but for which no data have been collected so far, must be considered, namely the front effects of the various constituents of the eluant. In column chromatography it is usual to wash the column with the eluant in order to have a certain ionic form of the resin and a certain solution in which the ions to be studied will be placed. In ion exchange paper chromatography it is possible to convert the paper to a certain form, however the sample to be run is usually placed on a dry paper and the solvent front is then allowed to run over it. If some constituents of the solvent are then retained by the ion exchange paper, the ions placed on the paper will not be in contact with the bulk eluant but with the solvent at the liquid front which may be altered by moving over the paper. In order to assess the magnitude of such front phenomena we have carried out a number of typical elution experiments in which the heights of the fronts were recorded.

EXPERIMENTAL

Amberlite resin papers SA-2 and SB-2 were washed twice with 2 *N* HCl and distilled water and then converted to the required form by leaving for 30 min in a normal solution of the required salt, washing with water and repeating the equilibration. The paper was then air dried overnight and developed by the ascending method in small volume jars. Hydrogen ions were detected by spraying with universal indicator and the other ions with suitable reagents or by their colour. Usually very sharp fronts were obtained which were readily reproducible.

RESULTS

(*i*) *Monovalent ion–monovalent ion equilibria*

Fig. 1 shows the H^+ ion front when the chloride form of the anionic SB-2 paper is developed with HCl solutions. There is practically no retention of H^+ ions and the H^+ front moves with or closely behind the solvent front. Fig. 2 shows the movement of the H^+ front when the sodium form of the cationic SA-2 paper is developed with HCl. The sodium ions are eluted and replaced by H^+ ions. Below 0.5 *N* HCl the H^+ front is surprisingly low and ions which are chromatographed in such a system and which are not very strongly adsorbed will move in a neutral medium and probably never come in contact with the HCl.

(*ii*) *Monovalent ion–divalent ion equilibria*

We chose as typical example the movement of a Ni^{++} front on the H^+ form of the sulphonic SA-2 paper, because Ni^{++} is little complexed in HCl and readily detected with ammonium sulphide. Fig. 3 shows the movement of the Ni^{++} front in the presence of varying amounts of HCl in the solvent. Even with 1 *N* $NiCl_2$ in the presence of 3 *N* HCl (which should displace the Ni^{++} ions considerably) the R_F value of the Ni^{++} front is only about 0.7. We should also like to emphasise that with very dilute solutions, *e.g.* 0.1 *N* Ni^{++} in the absence of H^+ in the solvent, the nickel hardly moves along the paper.

Another interesting example of a divalent–monovalent equilibrium is the movement of $H_2SO_4^-$ on the neutral sulphate form of the SB-2 paper. The sulphonic acid converts the neutral sulphate into HSO_4^- and as shown in Fig. 4 the H^+ front in very dilute solutions moves to about R_F 0.1 leaving the upper portion of the paper with water as the eluant. Here we also noted the curious behaviour of Fe^{+++} when chromatographed in this system. Fe^{+++} yields a narrow compressed band which coincides with the H^+ front. Two spots of Fe^{+++} placed 1 cm above each other were found at the same height after development, *i.e.* it is impossible to speak of R_F values of Fe^{+++} under these conditions and evidently the movement of Fe^{+++} on the resin paper will bear no relation to the behaviour of Fe^{+++} in batch or column experiments.

The oxalate form of the SB-2 paper developed with oxalic acid behaves similarly. The undissociated H_2Ox is adsorbed on the resin and the H^+ front is still lower than with the sulphate form and sulphuric acid as shown in Fig. 5. Another example, namely the movement of chromate on the chloride form of SB-2 paper, is given in Fig. 6.

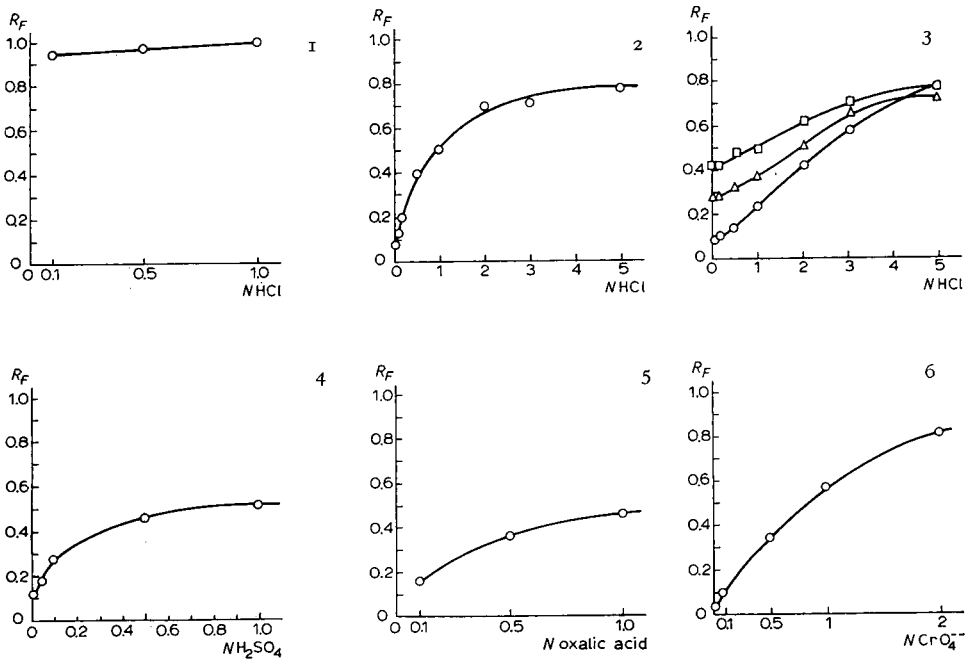

Fig. 1. The R_F values of the H^+ front plotted against HCl concentration on the Cl^- form of the basic SB-2 paper.

Fig. 2. The R_F values of the H^+ front plotted against the HCl concentration on the Na^+ form of the acidic SA-2 paper.

Fig. 3. The R_F values of Ni^{++} front plotted against the concentration of HCl (*i.e.* mixtures $NiCl_2$ and HCl as solvent) on the H^+ form of the acidic SA-2 paper. O——O = 0.1 *N* $NiCl_2$, △——△ = 0.5 *N* $NiCl_2$, and □——□ = 1.0 *N* $NiCl_2$.

Fig. 4. The R_F values of the H^+ front plotted against the concentration of H_2SO_4 on the SO_4^{2-} form of the basic SB-2 paper.

Fig. 5. The R_F values of the H^+ front plotted against the concentration of oxalic acid on the oxalate form of the basic SB-2 paper.

Fig. 6. The R_F values of the CrO_4^{2-} front plotted against the concentration of K_2CrO_4 on the Cl^- form of the basic SB-2 paper.

DISCUSSION

For practical purposes we can offer a few suggestions to overcome these effects.

1. The front effect can be ignored for systems such as the HCl–chloride form of the SB-2 paper.

2. When the front moves sufficiently fast, the spots to be chromatographed can be placed behind the front, *i.e.* the solvent is allowed to run for 5 or 10 cm and the spot is then placed at a measured distance behind the solvent front and this distance is subtracted when R_F values are determined.

3. When the front moves slowly, as for example with oxalate buffers of low

ionic strength, it would appear impossible to obtain equilibrium conditions by the usual development techniques. The technique of placing the samples behind the liquid front can be applied if the solvent is allowed to run over the paper by descending development for a considerable time (*e.g.* overnight) and if experimental evidence is obtained to show that the sample is really placed behind the front.

4. One useful criterion for suitable development conditions may be found in the shape of the developed spot. It should be somewhat larger than the original spot owing to diffusion etc. but should have retained the approximate shape of the original zone. Elongated comets or compressed narrow zones should be taken as evidence for non-equilibrium conditions and the technique should be suitably modified.

SUMMARY

The importance of the frontal separation of the eluants in ion exchange paper chromatography is demonstrated and several examples are discussed.

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HIGH- AND LOW-VOLTAGE PAPER ELECTROPHORETIC INVESTIGATION OF THE SOLUTION CHEMISTRY OF MERCURIC IONS IN NITRIC ACID

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A knowledge of the nature of the ionic species of an element present in aqueous solution which affects many of the physical and chemical properties of the solution[1] is of considerable interest in determining the method to be employed for the separation of the element in question and its quantitative isolation, as well as in the interpretation of the reactions or processes which take place in such a medium. Recently anion exchange chromatography[2–4] and solvent extraction[5] techniques have been employed as tools for investigating the state of combination of different elements in aqueous solution. For metal ions which have strong affinity for the ligands present in the aqueous system, thus forming stable complex species, the indications given by adsorption studies on anion exchangers may be reliable. But, as has already been pointed out by WAKI[4], "it is often observed that a metallic element may be significantly adsorbed on an anion exchange resin even from a solution in which the presence of any complex anion seems hardly to be considered". It is believed that the resin is an extremely concentrated solution of electrolytes[5] with low effective dielectric constants[6] and where the water has less effect[7] may bind to itself the cations or complex ions of low stability by converting them into anionic species. Similar reasoning can be extended to the solvent extraction systems using long-chain aliphatic amines, where the electrolyte is concentrated into the organic phase of very low dielectric constant; the amines are there as binders for the anionic species and thus serve as stabilizers of the complex anions.

The advantage of paper electrophoresis as an easy and valuable tool for the study of the nature of the ionic species present in a solution, as well as in the isolation of pure species for the study of their other properties, has recently been recognized[8–10] and the technique has already been applied to the study of the solution chemistry of many elements.

Mercuric nitrate, like the sulphate and perchlorate, is known to be ionized and highly dissociated in solution[11]. Furthermore, the nitrate ion, next to the perchlorate ion, is a ligand which is very reluctant to form covalent bonds with an element. Recently, however, the existence of $[Hg(NO_3)_3]^-$ and $[Hg(NO_3)_4]^{2-}$ in solution has been concluded from adsorption studies[4]. If the existence of such anionic species of mercury in 0.2 N HNO_3, as reported by CHOI AND TUCK[5], is accepted, then the exchange between Hg_2^{2+} and Hg^{2+} should be slow[12], while the contrary has been reported[13]. We therefore thought it of interest to investigate the nature of the ionic

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species furnished by Hg^{2+} in nitric acid solutions by paper electrophoresis, which permits a direct examination of the species without introduction of undesirable foreign substances in the system under study.

EXPERIMENTAL

The mercuric nitrate solution, tagged with ^{203}Hg (half-life = 47 days and specific activity 128.5 mC/g), used in these studies was supplied by C.E.A., Saclay. This solution was evaporated several times with nitric acid and finally redissolved in nitric acid of the desired strength. The solution was diluted so as to have suitable activity for paper electrophoretic studies. The concentration of nitric acid in the solution to be tested and that in the electrolyte was always kept the same. Studies were made in the concentration range 0.2–8 N HNO_3. In order to see how the mercuric species in relatively concentrated nitric acid behaves when placed in nitric acid of lower concentration, the solution of ^{203}Hg in 8 N HNO_3 was examined by electrophoresis in electrolytes of varying concentrations.

The solution was examined both by high- and low-voltage electrophoresis. The glass-plate (30 × 8 cm) technique described by LEDERER AND WARD[14] was employed for low-voltage electrophoresis. High-voltage electrophoresis was carried out with a locally made high-voltage electrophoresis apparatus described by GROSS[15]. Arches No. 302 paper strips (2.7 cm wide of suitable length, 30 cm long for low-voltage and 44 cm long for high-voltage electrophoresis) were used. Electrophoretic migrations were also observed in each case on glass paper (Whatman No. GF/C) strips.

The position of the ^{203}Hg(II) species on the electropherogram was located by scanning with the Frieseke Hoepfner FH 452 automatic chromatogram scanner, using a 3 mm wide slit.

RESULTS AND DISCUSSION

The sequence of displacement of the mercuric species in different concentrations of nitric acid was the same under high- and low-voltage electrophoresis as well as on the electropherograms on glass paper and Arches 302. Because of the better displacements, only those results that are obtained with high-voltage electrophoresis using Arches 302 paper strips are shown in Figs. 1 and 2 as typical. It is seen that the mercuric ion has a greater cationic speed in the concentration range 0.2–4 N HNO_3, while the displacement in the electrolyte at higher concentrations becomes relatively smaller. We conclude, therefore, that in the concentration range 0.2–4 N HNO_3, Hg^{2+} exists as an aquocomplex $[Hg(H_2O)_4]^{2+}$, which combines with NO_3^- in higher concentrations of nitric acid to give $[Hg(H_2O)_3(NO_3)]^+$, a complex with a lower net positive charge. The presence of the neutral complex $[Hg(H_2O)_2(NO_3)_2]^0$ was not revealed by electrophoresis in the concentration range of nitric acid studied and may be due to its low stability constant[16]. It may exist in nitric acid of higher concentration. However, in disagreement with the results of anion exchange and solvent extraction studies, electrophoretic results did not show the presence of an anionic species formed by Hg^{2+} with NO_3^-, which has been reported to be very marked in dilute nitric acid[4,5]. It is considered, therefore, that the adsorption of Hg^{2+} on the anion exchanger and its extraction in methyldioctylamine solution in chloroform

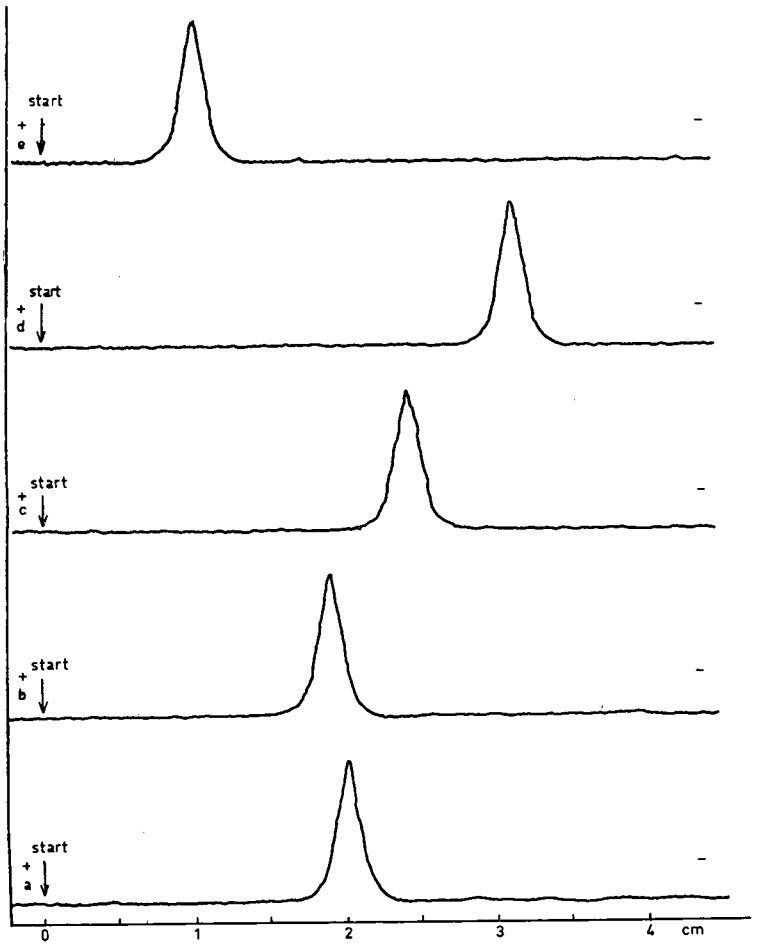

Fig. 1. High-voltage electropherograms of $^{203}Hg(NO_3)_2$ solutions on Arches 302 paper (44 × 2.7 cm). Conditions: (a) Electrolyte = 0.2 *N* HNO_3; 2500 V, 100 mA, 0.5 h; (b) Electrolyte = 0.5 *N* HNO_3; 1400 V, 125 mA, 1.0 h; (c) Electrolyte = 1.0 *N* HNO_3; 650 V, 150 mA, 1.0 h; and (d) Electrolyte = 2.0 *N* HNO_3; 620 V, 150 mA, 2.0 h.

might have been due to the high concentration of nitric acid in these systems of low dielectric constant and of low water content, favouring the stabilization of an otherwise unstable anionic species of mercuric ion in an aqueous system. The decreased adsorption as well as extraction with increase in the concentration of nitric acid may be accounted for by the saturation of the adsorbing or extracting system by several layers of undissociated nitric acid. This prevents the Hg^{2+} from coming in contact with the resin or the amine which contributed to stabilization of the anionic species in a lower concentration of the acid.

The electrophoretic mobility of Hg(II) in dilute nitric acid was almost the same as that of Hg(II) dissolved in 8 *N* HNO_3 electrophorised in the same electrolyte. This indicates the labile nature of the nitratocomplexes formed by Hg(II).

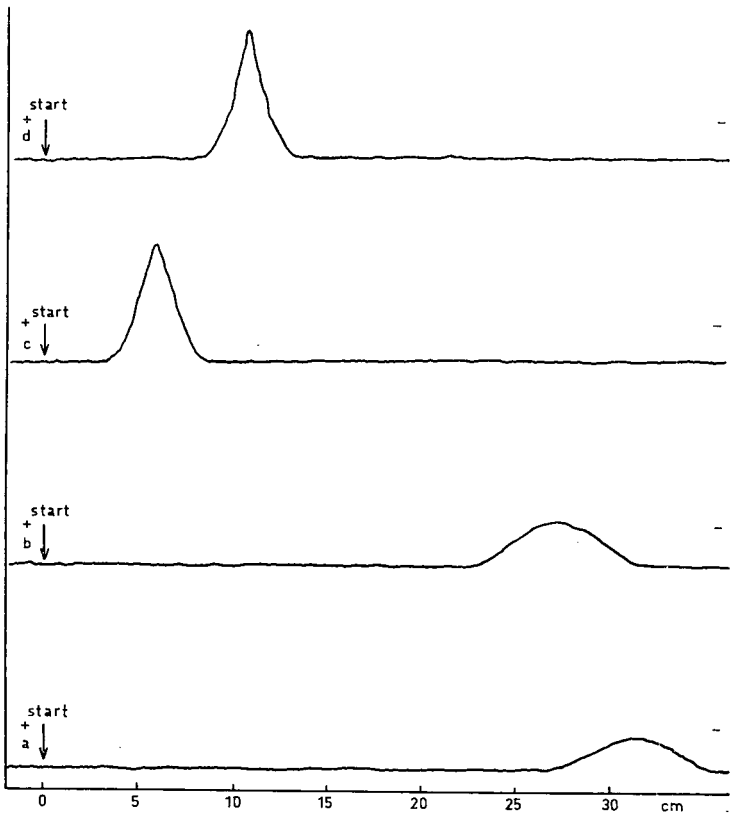

Fig. 2. High-voltage electropherograms of $^{203}Hg(NO_3)_2$ solutions on Arches 302 paper (44 × 2.7 cm). Conditions: (a) Electrolyte = 2.5 *N* HNO_3; 580 V, 150 mA, 0.5 h; (b) Electrolyte = 4 *N* HNO_3; 460 V, 110 mA, 0.5 h; (c) Electrolyte = 5 *N* HNO_3; 330 V, 140 mA, 2.5 h; (d) Electrolyte = 6 *N* HNO_3; 340 V, 150 mA, 2.5 h; and (e) Electrolyte = 8 *N* HNO_3; 130 V, 130 mA, 1.0 h.

SUMMARY

The nature of ionic species furnished by Hg^{2+} in nitric acid has been examined by paper electrophoresis in the concentration range 0.2–8 *N* HNO_3. Only cationic species $[Hg(H_2O)_4]^{2+}$ and $[Hg(H_2O)_3(NO_3)]^+$ were found in the concentration range 0.2–4 *N* and 4–8 *N*, respectively. The solvent extraction and anion exchange adsorption results reported in literature have been explained by taking into consideration the properties of the extracting and the adsorbing system.

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CORRELATIONS OF ELECTROPHORETIC MOBILITIES IN BORATE BUFFER WITH STRUCTURAL FACTORS OF SOME FLAVONOID COMPOUNDS

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INTRODUCTION

The versatility of two-dimensional paper chromatography for studying the distribution and structure of flavonoid compounds, and also the elegant use of thin-layer chromatography for resolving mixtures of their derivatives has been widely demonstrated. By comparison, no systematic data exist on their electrophoretic mobilities (*cf.* refs. 1, 2). The reason for this must perhaps be sought in the observation that chromatographic effects on borate-impregnated paper closely parallel many of the coordination effects commonly achieved in similar systems by paper electrophoresis. Electrophoresis, nevertheless, is capable of revealing many small structural differences between compounds, as is shown by the present systematic attempts at correlating paper electrophoretic behaviour with flavonoid structure.

EXPERIMENTAL AND RESULTS

Origin of compounds

Most substances used are natural compounds or their synthetic conversion products. The majority were previously described by ROUX[3] and ROUX, MAIHS AND PAULUS[4]. Eriodictyol was kindly supplied by Dr. B. H. KOEPPEN, Stellenbosch University, Stellenbosch, Cape. Similarly, pinocembrin, pinobanksin, chrysin and tectochrysin, originating from various *Pinus* spp. (*cf.* ref. 5) were supplied by Prof. H. ERDTMAN, Royal Technical University, Stockholm. Kaempferol, (+)-aromadendrin and (+)-afzelechin from *Eucalyptus calophylla*[6,7] were kindly supplied by Dr. W. E. HILLIS, C.S.I.R.O., Melbourne. (—)-Melacacidin and isomelacacidin were isolated by preparative paper chromatography from the heartwood of *Acacia melanoxylon*[8]. 4′,7-Dihydroxy-, 7-hydroxy- and 4′-hydroxyflavanones were prepared by synthesis[9,10].

Electrophoretic method

Mobilities were measured by horizontal paper electrophoresis using a Vokam power supply (Shandon Scientific Co.) and Schleicher and Schüll 2043 (4 × 41 cm) paper. A constant current of 0.31 mA/cm width of paper was applied (4 × 4 cm papers requiring a total of 5 mA) for 6 h using borate buffer pH 8.8 (12.6 g/l sodium borate, 3.1 g/l boric acid). The distance of anodic migration was measured from the origin to the centre of the band. Bands were located under U.V. light or by spraying with

ammoniacal silver nitrate, *bis*-diazotized benzidine, or toluene-*p*-sulphonic acid, depending on the structure of the flavonoid (*cf.* ROUX *et al.*[3,4,11,12]). An average of two runs was used to calculate the migration distance. (+)-Catechin was used as reference compound with each run, and relative mobilities were expressed with (+)-catechin as unity.

Flavonoid compounds were made up in buffer just prior to the start of each run. In some cases (flavonols) a trace of ethanol was added to promote solubility.

Where ammoniacal silver nitrate was used for spraying, colour development did not occur until the strips were washed with distilled water, probably due to masking of the reducing (*ortho*-hydroxyphenolic) groups by borate ions.

Results

The relative mobilities (M) of the flavonoid compounds are given in Table I. Their stereochemistry[13] is as shown, but where they are racemates, only one enantiomer corresponding to the 2 *R* form of 2,3-*trans*-flavan-3-ols [(+)-catechin] is shown.

DISCUSSION

Consideration of structures I–XXXVI suggests that co-ordination of the borate ion might occur (a) at vicinal hydroxyls which may either be phenolic (3',4'- or 7,8-positions) or aliphatic (3,4-position); (b) with the 5-hydroxyl and 4-carbonyl positions as in certain flavones, flavanones, flavonols and dihydroflavonols; (c) with the 3-hydroxyl and 4-carbonyl as in dihydroflavonols (flavanonols) or flavonols.

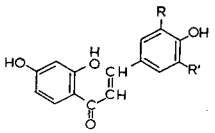
The effects of such co-ordination on electrophoretic mobility, where operative, are considered below, and further consideration of stereochemically related compounds show that other structural factors affect mobility to a variable degree.

Effects of possible co-ordination sites

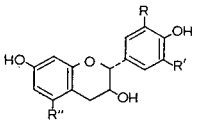
(*a*) *Vicinal hydroxyl groups.* Comparison of the flavonols, kaempferol (IX) (0.06) and quercetin (X) (0.25); of the dihydroflavonols, aromadendrin (XIV) (0.99) and taxifolin (XV) (1.64); of the chalcones, dahlia chalcone (I) (0.16) and butein (II) (0.33); and of the flavan-3-ols, (+)-afzelechin (XXI) (0.33) and (+)-catechin (XXII) (1.00), shows that provision of a phenolic *ortho*-hydroxyl system through introduction of a 3'-hydroxyl produces enhanced relative mobility ($\Delta M = +0.17$ to $+0.67$) irrespective of the type of compound under consideration. Similarly comparison of (+)-leucofisetinidin (XXIV) (1.05), with one (3',4') phenolic co-ordinating position, and isomelacacidin (XXXV) (1.64), with two (3',4' and 7,8) such positions, illustrates a similar ($\Delta M = +0.59$) effect on the introduction of additional vicinal phenolic hydroxyls, both compounds having a 3,4-*trans*-glycol grouping which exerts no effect (see below).

Examination of the mobilities of isomelacacidin (XXXV) (1.64) and (—)-melacacidin (XXXIV) (1.71) shows a small but significant difference in mobility which may be ascribed to additional co-ordination with the 3,4-*cis*-glycol grouping of the latter compared with the absence of co-ordination with borate at the 3,4-*trans*-glycol group of the former (see below). These compounds are identical in all other respects, both having the 2,3-*cis*-configuration of substituents.

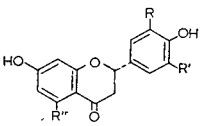
(*b*) *5-Hydroxyl and 4-carbonyl position.* Correlation of the mobilities of pairs



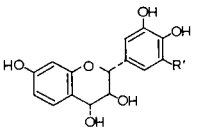
I Dahlia chalcone (R=R′=H)
II Butein (R=OH, R′=H)
III Robtein (R=R′=OH)



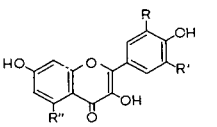
XIX Fisetinidol (R=OH, R′=R″=H)
XX Robinetinidol (R=R′=OH, R″=H)
XXI Afzelechin (R=R′=H, R″=OH)
XXII Catechin (R=R″=OH, R′=H)
XXIII Gallocatechin (R=R′=R″=OH)



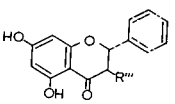
IV Butin (R=OH, R′=R″=H)
V Robtin (R=R′=OH, R″=H)
VI Eriodictyol (R=R″=OH, R′=H)



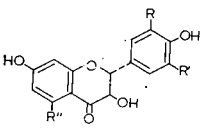
XXIV Leucofisetinidin (R′=H)
XXV Leucorobinetinidin (R′=OH)



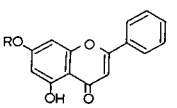
VII Fisetin (R=OH, R′=R″=H)
VIII Robinetin (R=R′=OH, R″=H)
IX Kaempferol (R=R′=H, R″=OH)
X Quercetin (R=R″=OH, R′=H)
XI Myricetin (R=R′=R″=OH)



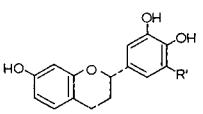
XXVI Pinocembrin (R‴=H)
XXVII Pinobanksin (R‴=OH)



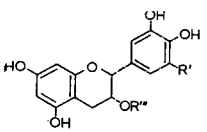
XII Fustin (R=OH, R′=R″=H)
XIII Dihydrorobinetin (R=R′=OH, R″=H)
XIV Aromadendrin (R=R′=H, R″=OH)
XV Taxifolin (R=R″=OH, R′=H)
XVI Ampeloptin (R=R′=R″=OH)



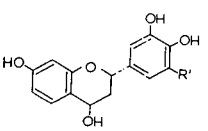
XXVIII Chrysin (R=H)
XXIX Tectochrysin (R=CH_3)



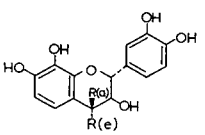
XVII



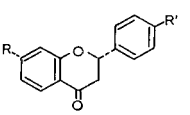
XXX Epicatechin (R′=R‴=H)
XXXI Epigallocatechin (R′=OH, R‴=H)
XXXII Epicatechin gallate (R′=H, R‴=galloyl)
XXXIII Epigallocatechin gallate (R′=OH, R‴=galloyl)



XVIII



XXXIV Melacacidin ($R_{(a)}$=H, $R_{(e)}$=OH)
XXXV Isomelacacidin ($R_{(a)}$=OH, $R_{(e)}$=H)



XXXVI

TABLE I

RELATIVE MOBILITIES (M)* OF FLAVONOID COMPOUNDS IN A SODIUM BORATE–BORIC ACID BUFFER

Compound	*M**	*Compound*	*M**
Chalcones		*2,3-trans-Flavan-3-ols*	
Dahlia chalcone (I)	0.16	Fisetinidol (XIX)	1.04
Butein (II)	0.33	Robinetinidol (XX)	0.96
Robtein (III)	0.17	Afzelechin (XXI)	0.33
		Catechin (XXII)	1.00
		Gallocatechin (XXIII)	0.93
Flavanones		*2,3-trans-Flavan-3,4-trans-diols*	
Butin (IV)	1.72	Leucofisetinidin (XXIV)	1.05
Robtin (V)	1.49	Leucorobinetinidin (XXV)	0.96
Eriodictyol (VI)	1.54		
Flavonols		*Flavonoids with unsubstituted B-ring*	
Fisetin (VII)	0.38	Pinocembrin (XXVI)	0.85
Robinetin (VIII)	0.24	Pinobanksin (XXVII)	0.93
Kaempferol (IX)	0.06	Chrysin (XXVIII)	0.11
Quercetin (X)	0.25	Tectochrysin (XXIX)	0.00
Myricetin (XI)	0.12		
Dihydroflavonols		*2,3-cis-Flavan-3-ols*	
Fustin (XII)	1.85	Epicatechin (XXX)	0.92
Dihydrorobinetin (XIII)	1.82	Epigallocatechin (XXXI)	0.80
Aromadendrin (XIV)	0.99	Epicatechin gallate (XXXII)	1.46
Taxifolin (XV)	1.64	Epigallocatechin gallate (XXXIII)	1.38
Ampeloptin (XVI)	1.48		
Flavans		*2,3-cis-Flavan-3,4-diols*	
3′,4′,5′,7-Tetrahydroxy (XVII, R′ = OH)	0.84	Melacacidin (XXXIV)	1.71
3′,4′,7-Trihydroxy (XVII, R′ = H)	0.97	Isomelacacidin (XXXV)	1.64
Flavan-4β-ols		*Flavanones with low degree of substitution*	
3′,4′,5′,7-Tetrahydroxy (XVIII, R′ = OH)	0.88	4′,7-Dihydroxy (XXXVI, R = R′ = OH)	0.84
3′,4′,7-Trihydroxy (XVIII, R′ = H)	1.00	7-Hydroxy (XXXVI, R = OH, R′ = H)	0.95
		4′-Hydroxy (XXXVI, R = H, R′ = OH)	0.00

* Mobilities relative to that of (+)-catechin.

of flavanonols, fustin (XII) (1.85) and taxifolin (XV) (1.64), dihydrorobinetin (XIII) (1.82) and ampeloptin (XVI) (1.48); of the flavonols, fisetin (VII) (0.38) and quercetin (X) (0.25), robinetin (VIII) (0.24) and myricetin (XI) (0.12); and of the flavanones, butin (IV) (1.72) and eriodictyol (VI) (1.54), shows that in each instance where a 5-hydroxyl is introduced (latter compound) a reduction ($\Delta M = -0.12$ to -0.34) instead of an anticipated increase in mobility is obtained. This effect is discussed below.

(*c*) *3-Hydroxyl and 4-carbonyl position.* Mobilities of compounds containing the 4-carbonyl but having the 3-hydroxyl either absent (flavanone) or present (flavanonol) *e.g.* butin (IV) (1.72) and fustin (XII) (1.85), robtin (V) (1.49) and dihydrorobinetin (XIII) (1.82), eriodictyol (VI) (1.54) and taxifolin (XV) (1.64), pinocembrin (XXVI) (0.85) and pinobanksin (XXVII) (0.93), show an increase ($\Delta M = +0.08$ to $+0.33$) with the availability of this possible co-ordination position. However, the effect is more likely to be related to an "affinity factor" (see below).

Lack of co-ordination with borate in both the latter positions is perhaps surprising considering that aluminium salts form very stable complexes with 5-hydroxy- and 3-hydroxy-flavanones[14], although in acid medium.

Hydroxylation at the 4'-position

The relative mobilities of pinobanksin (XXVII) (0.93) and (+)-aromadendrin (XIV) (0.99) show that introduction of a hydroxyl in the 4'-position in flavanonols causes a small increase ($\Delta M = +0.06$). This might be due to partial dissociation of the 4'-hydroxyl (compare effect of strong dissociation of the 7-hydroxyl due to its *para*-position to the 4-carbonyl in flavanones and flavanonols). However, for the flavanones, 7-hydroxyflavanone (XXXVI, R = OH, R' = H) (0.95) and 4',7-dihydroxyflavanone (XXXVI, R = R' = OH) (0.84), introduction of a 4'-hydroxyl has the opposite effect ($\Delta M = -0.11$) on mobility.

Hydroxylation at the 5'-position in the presence of 3',4'-dihydroxyls

Comparison of the stereochemically related (where applicable) pairs of flavan-3-ols, (+)-catechin (XXII) (1.00) and (+)-gallocatechin (XXIII) (0.93), (—)-epicatechin (XXX) (0.92) and (—)-epigallocatechin (XXXI) (0.80), (—)-epicatechin gallate (XXXII) (1.46) and (—)-epigallocatechin gallate (XXXIII) (1.38), (—)-fisetinidol (XIX) (1.04) and (—)-robinetinidol (XX) (0.96); of flavan-4β-ols, 3',4',7-trihydroxy (XVIII, R' = H) (1.00) and 3',4',5',7-tetrahydroxy (XVIII, R' = OH) (0.88); of flavans, 3',4',7-trihydroxy (XVII, R' = H) (0.97) and 3',4',5',7-tetrahydroxy (XVII, R' = OH) (0.84); of flavan-3,4-diols, (+)-leucofisetinidin (XXIV) (1.05) and (+)-leucorobinetinidin (XXV) (0.96); of dihydroflavonols, (±)-fustin (XII) (1.85) and (±)-dihydrorobinetin (XIII) (1.82), (±)-taxifolin (XV) (1.64) and (±)-ampeloptin (XVI) (1.48); of flavonols, fisetin (VII) (0.38) and robinetin (VIII) (0.24), quercetin (X) (0.25) and myricetin (XI) (0.12); of flavanones, (±)-butin (IV) (1.72) and (±)-robtin (V) (1.49); and of the chalcones, butein (II) (0.33) and robtein (III) (0.17), shows that introduction of a 5'-hydroxyl in the presence of 3',4'-dihydroxyls causes a variable ($\Delta M = -0.03$ to -0.22) reduction in mobility.

Hydroxylation at the 3'-position in the presence of a 4'-hydroxyl

Comparison of the stereochemically related (where applicable) pairs of flavan-3-

ols, (+)-afzelechin (XXI) (0.33) and (+)-catechin (XXII) (1.00); of dihydroflavonols, (+)-aromadendrin (XIV) (0.99) and (+)-taxifolin (XV) (1.64); of flavonols, kaempferol (IX) (0.06) and quercetin (X) (0.25); and of the chalcones, dahlia chalcone (I) (0.16) and butein (II) (0.33) show a large positive effect for the non-planar ($\Delta M = +0.65, +0.77$) and a lesser effect ($\Delta M = +0.17, +0.19$) for planar flavonoids on introduction of the 3'-hydroxyl in the presence of the 4'-hydroxyl.

These positive effects on mobilities of the 3'-hydroxyl in the presence of the 4'-hydroxyl contrast with the negative effect on mobility with the introduction of the equivalent 5'-hydroxyl in the presence of 3'- and 4'-hydroxyls, and must be due almost exclusively to the co-ordination with the borate ion to form a negatively charged complex.

Simultaneous hydroxylation at 3',4'-positions

The above theory is supported by the observation that where two hydroxyls are introduced simultaneously in the *ortho*-position, as in the comparison (+)-pinobanksin XXVII (0.93) and (±)-taxifolin (XV) (1.64); (—)-pinocembrin (XXVI) (0.85) and (±)-eriodictyol (VI) (1.54), the increase in mobility ($\Delta M = +0.74, +0.68$) is of the same order as for the non-planar flavonoids above where the 3'-hydroxyl is introduced.

Hydroxylation at the 8-position in the presence of a 7-hydroxyl

An effect, almost of the same magnitude, is observed when introducing a second *ortho*-hydroxyl group through 8-hydroxylation, in the comparison of (+)-leucofisetinidin (XXIV) (1.05) with isomelacacidin (XXXV) (1.64) ($\Delta M = +0.59$), and the increased mobility must again be due to co-ordination with an additional borate ion only.

Hydroxylation at the 5-position

In order to study this effect independently of others, comparison must be made between mobilities of stereochemically related flavan-3-ols, (—)-robinetinidol (XX) (0.96) and (+)-gallocatechin (XXIII) (0.93); (—)-fisetinidol (XIX) (1.04) and (+)-catechin (XXII) (1.00). Introduction of a 5-hydroxyl into flavan-3-ols thus has a negative effect on mobility ($\Delta M = -0.03, -0.04$) similar to those evident from previous considerations of co-ordination sites where introduction of a 5-hydroxyl into a flavanone has a somewhat larger effect ($\Delta M = -0.18$). Also introduction of the 5-hydroxyl in the presence of a 4-carbonyl plus 3-hydroxyl has an effect of similar magnitude in dihydroflavonols ($\Delta M = -0.21, -0.34$) and in flavonols ($\Delta M = -0.13, -0.12$).

The 5-hydroxyl therefore has a retarding effect on mobility which appears to be accentuated by the presence of a 4-carbonyl or a 4-carbonyl plus 3-hydroxyl. From this it may be concluded that no complexing with borate ions occurs, and the effect is rather similar to the introduction of a 5'-hydroxyl in the presence of the 3',4'-dihydroxyl arrangement where no further complexing is possible as in the flavan-3-ols ($\Delta M = -0.07, -0.08$), dihydroflavonols (—0.16, —0.03), flavonols (—0.14, —0.12) and flavanones (—0.23).

The higher relative mobility of flavanones, flavonols and dihydroflavonols of the "resorcinol" series, when compared with the equivalent compounds of the

"phloroglucinol" series (*cf.* Table I) might be due in part also to a reduction in the acidic properties of the 7-hydroxyl (flavanones and dihydroflavonols) or 7- plus 4-hydroxyls (flavonols) due to strong hydrogen bonding between the 5-hydroxyl and 4-carbonyl groups in these flavonoids of the "phloroglucinol" series[15] (*cf.* discussion of the effects on mobility of ionization induced by the 4-carbonyl group).

Hydroxylation at the 3-position

From the mobility data in Table I, the introduction of a 3-hydroxyl into flavans as in the pairs, (±)-3′,4′,5′,7-tetrahydroxyflavan (XVII, R′ = OH) (0.84) and (—)-robinetinidol (XX) (0.96); (±)-3′,4′,7-trihydroxyflavan (XVII, R′ = H) (0.97) and (—)-fisetinidol (XIX) (1.04), or into flavan-4β-ols as in the pairs, 3′,4′,5′,7-tetrahydroxyflavan-4β-ol (XVIII, R′ = OH) (0.88) and (+)-leucorobinetinidin (XXV) (0.96); 3′,4′,7-trihydroxyflavan-4β-ol (XVIII, R′ = H) (1.00) and (+)-leucofisetinidin (XXIV) (1.05), may be shown to produce a positive mobility (ΔM = +0.05 to +0.11).

Similar effects are evident when comparing similar flavanone pairs, (+)-eriodictyol (VI) (1.54) and (±)-taxifolin (XV) (1.64); (±)-robtin (V) (1.49) and (±)-dihydrorobinetin (XIII) (1.82); (±)-butin (IV) (1.72) and (±)-fustin (XII) (1.85); and pinocembrin (XXVI) (0.85) and pinobanksin (XXVII) (0.93), the degree of increase of mobility being exceedingly variable (+0.08 to +0.33). Notable is the exceptionally large increase, ΔM = +0.33, with introduction of the 3-hydroxyl into robtin.

The variable degree of increase of mobility, and the relatively small increases (ΔM = +0.08 to +0.13) in most cases whether in the presence or absence of the 4-carbonyl, suggests that this effect is entirely due to a reduction of affinity for cellulose as demonstrated by paper chromatography in 2 % acetic acid[3,4].

Hydroxylation at the 4β-position

Comparison of the mobilities of the pairs, (±)-3′,4′,5′,7-tetrahydroxyflavan (XVII, R′ = OH) (0.84) and (±)-3′,4′,5′,7-tetrahydroxyflavan-4β-ol (XVIII, R′ = OH) (0.88), (±)-3′,4′,7-trihydroxyflavan (XVII, R′ = H) (0.97) and (±)-3′,4′,7-trihydroxyflavan-4β-ol (XVIII, R′ = H) (1.00) would indicate that the 4β-hydroxyl increases electrophoretic mobility, an effect again similar to that found on paper chromatography in aqueous medium[3,4].

This effect does not operate in all instances as in the stereochemically related pairs (—)-fisetinidol (XIX) (1.04) and (+)-leucofisetinidin (XXIV) (1.05); (—)-robinetinidol (XX) (0.96) and (+)-leucorobinetinidin (XXV) (0.96) no appreciable increase is observed for the same factor.

Carbonyl group at the 4-position

Comparison of 2,3-dihydroflavonols with flavan-3-ol analogues shows that introduction of a 4-carbonyl group greatly increases electrophoretic mobility. The magnitude of the increase may be judged from the pairs (+)-afzelechin (XXI) (0.33) and (+)-aromadendrin (XIV) (0.99); (+)-catechin (XXII) (1.00) and (±)-taxifolin (XV) (1.64); (+)-gallocatechin (XXIII) (0.93) and (±)-ampeloptin (XVI) (1.48); (—)-fisetinidol (XIX) (1.04) and (±)-fustin (XII) (1.85); and (—)-robinetinidol (XX) (0.96) and (±)-dihydrorobinetin (XIII) (1.82), where ΔM = +0.55 to +0.86.

That these differences are due exclusively to the introduction of the 4-carbonyl group is shown by the comparisons (±)-3′,4′,5′,7-tetrahydroxyflavan (XVII, R′ = OH) (0.84) and (±)-robtin (V) (1.49); (±)-3′,4′,7-trihydroxyflavan (XVII, R′ = H) (0.97) and (±)-butin (IV) (1.72), where the increases $\Delta M = +0.65, +0.75$ are of the same magnitude as above.

The induction of high mobility by the 4-carbonyl in flavanones and flavanonols is presumably due to the far stronger ionization in borate buffer of the 7-hydroxyl in these than in their flavan and flavan-3-ol analogues, due to its location *para* to the carbonyl group. The higher acidity of the 7-hydroxyl is, for example, shown in the selective alkylation of the appropriate polyhydroxyisoflavones in the synthesis of prunetin and santal[16].

Unequivocal proof of the above is afforded by comparison of 4′-hydroxyflavanone (XXXVI, R = H, R′ = OH) (0.00) with 4′,7-dihydroxyflavanone (XXXVI, R = R′ = OH) (0.84), and with 7-hydroxyflavanone (XXXVI, R = OH, R′ = H) (0.95), where the relatively high mobility in flavanones correlates with the simultaneous presence of the 7-hydroxyl group.

This large positive mobility effect is the opposite of the strong affinity effect (reduction of R_F) on paper chromatograms in aqueous medium, when the same pairs of compounds are compared[3,4].

Galloyl group at the 3-position

Comparison of the mobilities of the pairs, (—)-epicatechin (XXX) (0.92) and (—)-epicatechin gallate (XXXII) (1.46); (—)-epigallocatechin (XXXI) (0.80) and (—)-epigallocatechin gallate (XXXIII) (1.38), shows a large increase in mobility ($\Delta M = +0.54, +0.54$) due to galloylation of the 3-hydroxyl group.

The increase in mobility must be due to the introduction of an additional phenolic *ortho*-hydroxyl system (co-ordination position for borate ion), and is of the same order as when a second phenolic *ortho*-hydroxyl system is introduced into the flavan nucleus; compare (+)-leucofisetinidin (XXIV) (1.05) and isomelacacidin (XXXV) (1.64) ($\Delta M = +0.59$). The positive mobility on galloylation is the opposite of the chromatographic affinity effect in 2 % acetic acid where the R_F is reduced.

Stereochemical effects

(*a*) *2,3-cis and 2,3-trans configurations*. Comparison of the electrophoretic mobility of pairs of the above configurations, respectively: (—)-epicatechin (XXX) (0.92) and (+)-catechin (XXII) (1.00); (—)-epigallocatechin (XXXI) (0.80) and (+)-gallocatechin (XXIII) (0.93), shows that when the 3-hydroxyl occupies an *axial* position as in 2,3-*cis*-(epi)-flavan-3-ols the mobility is lower ($\Delta M = -0.08, -0.13$) than when it is *equatorial* as in the 2,3-*trans*-flavan-3-ols.

A parallel affinity effect is shown on chromatography in water or 2 % acetic acid[3,4].

(*b*) *3,4-cis- and 3,4-trans-configurations*. The effect of 3,4-*trans*-glycol groups on mobility in borate buffer may be examined by comparison of 2,3-*trans*-flavan-3-ol and 2,3-*trans*-flavan-3,4-*trans*-diol pairs, (—)-fisetinidol (XIX) (1.04) and (+)-leucofisetinidin (XXIV) (1.05); (—)-robinetinidol (XX) (0.96) and (+)-leucorobinetinidin (XXV) (0.96) where the 3,4-*trans*-glycol group does not apparently contribute to mobility.

However, comparison of the 2,3-*cis*-flavan-3,4-*trans*-diol and 2,3-*cis*-flavan-3,4-*cis*-diol pair, isomelacacidin (XXXV) (1.64) and (—)-melacacidin (XXXIV) (1.71) suggests that the 3,4-*cis*-diol grouping contributes to the mobility ($\Delta M = +0.07$), which is probably reduced by the greater affinity of (—)-melacadicin for cellulose[4]. These findings have been confirmed by previous electrophoretic work on the fully methylated ethers of 3,4-*cis*- and 3,4-*trans*-flavandiols, where only the 3,4-*cis*-forms have been shown to have anodic mobility in sodium borate (*cf.* DREWES AND ROUX[17]).

Correlation between mobility and planarity

The relatively low electrophoretic mobilities of the flavonols and chalcones compared with high mobilities of dihydroflavonols, flavanones and also flavan-3-ols, flavan-4-ols and flavan-3,4-diols (Table I) is probably due to the high affinity, resulting from planar structure, of the former compared with the latter group. A similar relationship has been shown in paper chromatography in aqueous medium[3,4,11,12].

Mobility of compounds lacking the ortho-dihydroxy system

Six non-planar compounds (Table I) which do not contain the complex-forming *ortho*-dihydroxy system, namely aromadendrin (XIV) (0.99), pinocembrin (XXVI) (0.85), pinobanksin (XXVII) (0.93), 4',7-dihydroxyflavanone (XXXVI, R = R' = OH) (0.84), 7-hydroxyflavanone (XXXVI, R = OH, R' = H) (0.95) and afzelechin (XXI) (0.33), show relatively high mobility in spite of the proved absence of other co-ordination sites. Amongst these, the high mobility of the flavanones (XIV) (XXVI) (XXVII) (XXXVI, R = R' = OH and R = OH, R' = H) must be ascribed to the strong ionization of the 7-hydroxyl induced by the 4-carbonyl in the boric acid–sodium borate buffer. In the absence of the carbonyl group as in afzelechin (XXI), the greatly reduced but still significant mobility must be due to the far weaker dissociation of 7- and/or 4'-hydroxyls.

The flavanone, chrysin (XXVIII), has mobility (0.11) in spite of the reduced degree of hydroxyl substitution, absence of co-ordinating positions, and high affinity for cellulose due to planarity. Mobility must, therefore, be due to the strong ionization of the 7-hydroxyl as above. In tectochrysin (XXIX), where this position is blocked by methoxylation, mobility drops to zero. Parallel conclusions have been drawn by JURD AND HOROWITZ[18] from examination of the spectral shifts of flavones in sodium acetate buffer.

CONCLUSIONS

Paper ionophoresis of 40 flavonoid compounds in boric acid–sodium borate buffer has enabled tentative correlations between certain structural factors and mobility. Vicinal phenolic hydroxyls and 3,4-*cis*-glycol systems enhance mobility due to complex formation with borate, but no complexing occurs at 3-hydroxy-4-carbonyl and 5-hydroxy-4-carbonyl sites. Strongly enhanced mobility is induced by the introduction of a 4-carbonyl due to strong ionization of the 7-hydroxyl group. Mobility is also enhanced by hydroxylation in the 3- and 4β-positions, and by galloylation of the 3-hydroxyl, but is retarded by hydroxylation in 5'- and 5-positions, 2,3-*cis*- as compared with 2,3-*trans*-arrangements of substituents, and strongly reduced by the overall planarity of the flavonoid unit. Mobility effects produced by hydroxylation in the

5-, 5'-, 4β- and 3-positions and by molecular planarity may be correlated with affinity phenomena for cellulose, but galloylation and introduction of a 4-carbonyl produce effects opposite to those found on paper chromatograms due to factors cited above. In general, hydroxylation in positions where they are capable of ionizing (7 and in some cases 4') or when of aliphatic character (3,4β), apparently enhances mobility, whereas introduction of undissociated hydroxyls (5',3 and in flavanones, 4') retards ionophoretic mobility, provided no simultaneous complexing with borate occurs in these positions.

The relative mobilities of flavonoids are of diagnostic value in establishing their identity, while many of the specific mobility effects in borate buffer might assist in resolving components in complex mixtures or in establishing their homogeneity.

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SUMMARY

Comparison of the electrophoretic mobilities of flavonoid compounds in sodium borate–boric acid buffer has shown that co-ordination of borate with phenolic *ortho*-hydroxyls and ionization of the 7-hydroxyl correlate with large increases in mobility, whereas overall planarity strongly reduces mobility. The effects of hydroxyl, carbonyl and galloyl substituents, and of *cis*- and *trans*-arrangements in the 2,3- and 4-positions are related to smaller differences in mobility. These differences in mobility are of diagnostic value.

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Notes

Direct measurement of column hold-up in gas–liquid chromatography when using ionization detectors

To determine the absolute retention data the "gas hold-up"[1] in gas–liquid chromatography (GLC) must be evaluated. When using detectors which respond to permanent gases the gas hold-up can be directly measured from the air peak corresponding to the elution of a non-absorbed air sample. Since ionization detectors are insensitive to permanent gases the direct "air peak" method is not applicable under the conditions prevailing in a conventional GLC apparatus. Therefore, several indirect methods[2–7] have been proposed based on the linear relationship of the logarithm of the corrected retention time and the carbon number of a homologous series[8]. Using this linear relationship the gas hold-up has been evaluated as a "mathematical air peak"[2,3] and as a "calculated dead time[4–7] from the retention times of at least three members of a homologous series. The only direct method reported in the literature is that of "methane peak", which is considered unreliable as a generally applicable method[7,9] because of the small solubility of methane in organic solvents.

During the present work it has been found that when the carrier gas is presaturated with a low volatility organic solvent, negative air peaks can be readily detected with a hydrogen flame ionization detector. By measuring the retention time of the air peak and making allowance for the vapour pressure of the solvent, the gas hold-up of the apparatus can be directly determined. The gas hold-up of a packed column was directly determined by the above air peak method. Fig. 1 shows the injection points, the negative air peaks and the *n*-butane peaks for three identical vapour samples of *n*-butane–air mixture as detected with a hydrogen flame ionization detector. The carrier gas was presaturated with the solvent (*n*-decane) at the column temperature of 30.0°.

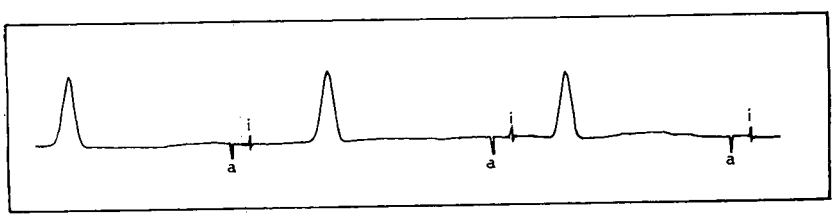

Fig. 1. Chromatogram of *n*-butane–air samples on *n*-decane showing injection points (i) and air peaks (a). Each sample is a 1.0 ml mixture containing 3.6×10^{-4} g *n*-butane.

The gas hold-up determined from the above method was compared with that estimated from a method recommended for capillary columns[1] and based on calculating the interstitial volume from the column characteristics. The method was modified for calculating the gas hold-up in the packed column in such a way that the

combined volume of the column, the sample injector and the detector was determined before packing the column by measuring the volume corresponding to the peak maximum of an organic solute in the dry nitrogen carrier gas and with no solvent or solid support in the column. The volume of the solvent was determined by an internal standard. The volume of the solid support was calculated from its density as determined by a specific gravity bottle. The results from the two methods differ by 2 %. The difference was mainly due to uncertainty in the effective volume of the solid support.

It is concluded that with hydrogen flame ionization detector it is now possible to determine directly, by air peak, the gas hold-up of GLC apparatus by presaturating the carrier gas with an organic solvent prior to the injection of the air sample. In the case of non-volatile solvents, the carrier gas may be saturated with any other suitable organic solvent of low volatility at the column temperature.

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Resolution of amino acids by gas chromatography*

The resolution of racemic mixtures of secondary *n*-alkanols by gas chromatography of the corresponding diastereoisomeric α-hydroxypropionates has been reported earlier[1,2]. The present communication describes a similar approach to the separation of α-amino acids.

The amino acids examined were chromatographed in the form of N-trifluoroacetyl (N-TFA) esters[3] of 2-*n*-alkanols, the latter serving to introduce an additional asymmetric center. The derivatives were prepared in nearly quantitative yields by esterification in the presence of HCl, and treating the resulting amino ester hydrochlorides in methylene chloride solution with excess trifluoroacetic anhydride at −20° with stirring. The reaction mixture was allowed to warm up to room temperature and left for 1 h.

* This work was performed under the sponsorships of the U.S. National Bureau of Standards.

Gas chromatography was carried out on capillary columns of 150 ft. length and 0.01 in. I.D., provided with a hydrogen flame ionization detector. The diastereoisomers derived from alanine, valine, leucine, isoleucine, proline, phenylalanine and glutamic acid could be separated under the experimental conditions given in Table I.

TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF THE DIASTEREOISOMERS OF N-TFA-AMINO ACID ESTERS

*N-TFA-Amino acid ester**		*Column***	*Temperature*§	*Relative retention time diethyl sebacate = 1*		$r_{LL/LD}$§§
Amino acid	*Alcohol*			*LL or DD*	*LD or DL*	
(±) Ala	(±) 2-Octanol	A	140	0.314	0.343	1.09
		A	160	0.421	0.440	1.05
		B	150	0.210	0.227	1.08
	± 2-Butanol	A	140	0.109	0.114	1.04
(±) Val	(±) 2-Octanol	A	140	0.433	0.466	1.08
		A	160	0.525	0.555	1.06
		B	150	0.251	0.263	1.05
	± 2-Butanol	A	140	0.143	0.147	1.03
(±) Leu	(±) 2-Octanol	A	140	0.576	0.615	1.07
		A	160	0.646	0.683	1.06
		B	150	0.385	0.401	1.04
(±) Pro	(±) 2-Octanol	A	140	1.351	1.490	1.10
		A	160	1.335	1.460	1.09
		B	150	0.890	0.955	1.08
(±) Phe	(±) 2-Octanol	A	140	3.10	3.26	1.05
		A	160	2.56	2.67	1.04
(±) Glu	± 2-Butanol	A	140	1.44	1.49	1.04

* Allocation of the peaks of the isoleucine derivatives (see text) has not yet been made; they overlap those of the N-TFA-leucine 2-octyl esters.

** A = capillary column (Perkin Elmer) FS-1265 coated with trifluoropropylmethyl polysiloxane; flow rate 1.5 ml N_2/min. B = capillary column (Perkin Elmer) coated with polypropylene glycol; flow rate 2.6 ml N_2/min.

§ The retention time of diethyl sebacate (in min) is: for 140° 12.1, for 150° 23.2, and for 160° 4.55.

§§ Ratio of the retention times of the diastereoisomers.

The degree of resolution obtained for some of the above compounds can be seen in Fig. 1. Isoleucine, which contains two asymmetric carbon atoms, can be resolved in the form of the N-TFA ester of 1-octanol, whereas the corresponding 2-*n*-octyl derivative gives four peaks as expected. Trifluoropropyl-methyl polysiloxane is preferred to polypropyleneglycol as stationary phase, because of the shorter analysis time and generally higher ratio between the retention times of the diastereoisomers. A temperature of 140° was found suitable for the separation of all derivatives studied, except for N-TFA-phenylalanine 2-octyl ester, which was better resolved at 180°.

In order to allocate the peaks, derivatives were prepared from optically active reagents. As in the case of α-alkanoyloxypropionates[1,2] the DL (or LD) compounds had

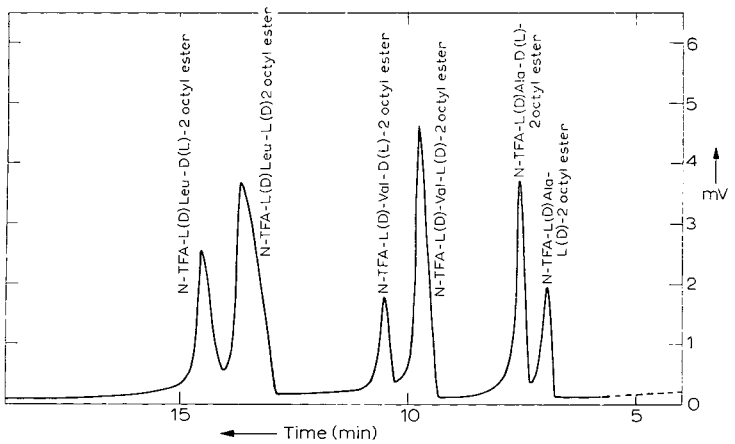

Fig. 1. Chromatogram of diastereoisomers of N-TFA-amino acid 2-octyl esters. Column, see Table I, column A; temperature 130°; flow rate 1.5 ml N_2/min.

in all cases a higher retention volume. Small amounts (1–5 %) of the second diastereoisomer found in the products of these syntheses are considered to result from incomplete steric homogeneity of the reagents.

For application of the method to amino acids, the N-TFA esters of which have relatively low retention times, 2-octanol is a convenient reagent, since its enantiomorphs are commercially available. 2-Butanol is suitable for amino acids, such as glutamic acid, which lead to compounds with high retention times. Modified types of diastereoisomeric derivatives, *e.g.*, amino acid methyl esters having an asymmetric centre in the group attached to the nitrogen atom, might be needed for extension of the resolutions to other natural amino acids.

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New procedure for packing alumina columns*

The conventional procedure[1] for preparing alumina columns in adsorption chromatography, *i.e.* by tapping the tube while the alumina is manually added in a fine stream, has several disadvantages. The method is laborious and requires continued attention, especially if large quantities of alumina are used. In addition, continued exposure of the alumina to the atmosphere may change its activity, owing to adsorbed moisture. The following procedure describes a technique which has many advantages over the usual method. It allows a column to be packed uniformly by sedimentation in a relatively dry atmosphere and with little or no supervision. As bonuses, the resultant zones are well defined, and execution of the chromatography is hastened, since elution is faster through this evenly-filled column.

The columns used are commercially available. They consist of a glass tube with a sintered glass disc and a teflon stopcock or reverse-tapered teflon stopcock (Fig. 1).

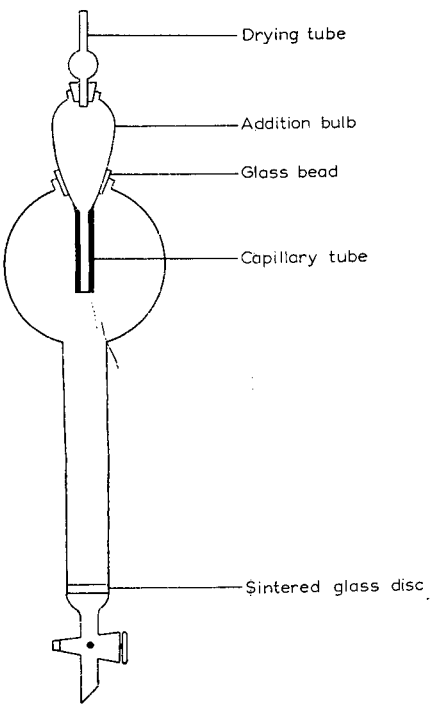

Fig. 1. Apparatus for packing alumina columns.

The stopcock is closed and the tube is filled to the bulb portion of the column with the desired liquid. Trapped air is completely removed by withdrawing a small volume of liquid through the stopcock. Alumina (100–200 mesh) is rapidly transferred to the addition bulb (Fig. 1) previously placed on the column. The addition bulb, protected then with a drying tube, is properly oriented to allow a continuous, fine stream of

* Contribution No. 312 of Central Research Laboratories, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota, U.S.A.

alumina to flow into the center of the column with resultant uniform distribution, wetting, and packing. The bulb's size is dictated by the quantity of adsorbent used; however, the diameter of the capillary tube on the bulb remains constant (2 mm I.D. for 100–200 mesh alumina affords an uninterrupted, fine stream). The capillary length should be such as to avoid contact with any upwardly displaced liquid. The addition requires little, or, usually, no monitoring*. Pressure equilibration between the column and bulb is accomplished by three glass beads, equally spaced and molded on the side of the bulb. After the addition is complete, the adsorbent is allowed to settle and the liquid is drawn off, until just above the alumina surface. The tube is repeatedly refilled with the liquid until the adsorbent no longer shows any tendency to settle. During this process, the alumina must *always* remain covered with liquid. The column is then covered with a layer of sand to insure a level alumina surface. Execution of the chromatography is performed in the usual manner.

In this laboratory, the ratio of the diameter of the alumina column to its height (not the height of the tube) has been kept as close to 1:5 as possible with the weight ratio of material to alumina about 1:30. Approximately 100 column adsorption chromatograms have been conducted in this manner, and zones were generally well defined, where visible. It has also been noted that the eluant passes through this column much more rapidly than through the hand-filled and tapped column.

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* A cone formed from glassine- or weighing-paper, may be substituted for the addition bulb, especially for small quantities (<30 g) of alumina. This modification is not recommended for larger quantities of adsorbent, owing to adsorption of moisture.

Reaction of α- and β-conidendrin with chromatographic solvents*

Two developing solvents widely used in the paper chromatographic separation of phenolic wood extractives, namely butanol–acetic acid–water (BAW) (60:15:25) and 2 % aqueous acetic acid, reacted with both α- and β-conidendrin[1] to form small quantities of reaction products. This was first observed in two-dimensional chromatography using BAW followed by 2 % acetic acid. In these solvent systems α-conidendrin

* Department of Forestry of Canada, Forest Products Research Branch Contribution Number P-71.

and its reaction products formed a single spot R_F 0.83 in BAW which in the 2 % acetic acid direction was resolved into two main spots R_F 0.00 (α-conidendrin) and R_F 0.50 together with a lesser spot R_F 0.70. Under similar conditions β-conidendrin had an R_F 0.86 in the first direction and produced only two spots R_F 0.00 (β-conidendrin) and R_F 0.53 in the second. In one-dimensional chromatography using 2 % acetic acid, α-conidendrin gave rise to three spots R_F 0.00, 0.50 and 0.70 and β-conidendrin to two spots R_F 0.00 and 0.58, although considerable streaking was evident in both cases. The larger amounts of reaction products detected when using two-dimensional BAW–2 % acetic acid is indicative of reaction with the acetic acid in both solvent systems. Because of the low concentration of the reaction products, detection was doubtful with diazotized sulfanilic acid (DSA)[2], but was positive using ferric ferricyanide reagent (FF)[3].

Small quantities of reaction products could be conveniently produced by treatment of either α- or β-conidendrin with acetic acid–water (1:1) at room temperature. No increase in the ratio of reaction products to the parent lactone after 12 h was noted indicating equilibrium had been established. These reaction mixtures were chromatographed in different solvent systems both before and after removal of the acetic acid and compared with chemically related compounds as shown in Table I. Attempts to isolate the R_F 0.50 compound from the α-conidendrin–acetic acid mixtures, either by low temperature vacuum distillation or lyophilization, resulted in only starting material and a small quantity of the R_F 0.70 compound, no R_F 0.50 remaining. Under similar conditions the β-conidendrin–acetic acid mixture produced mainly β-conidendrin and a weak spot R_F 0.58. Sufficient R_F 0.70 material was obtained for an infrared spectrum to be taken. A comparison of this spectrum with that of an authentic sample of α-conidendric acid[1] showed agreement of the major absorption peaks, which together with chromatographic data strongly indicated the R_F 0.70 spot to be α-conidendric acid. Thus, while it is still true that the quantitative conversion of α-conidendrin to its hydroxy acid must be done under strong hydrolyzing conditions[1], it is significant that detectable quantities of this acid can be obtained from the relatively mild conditions encountered with 2 % acetic acid at room temperature.

Further evidence of interconversion during paper chromatography was provided by the behaviour of pure α-conidendric acid in 2 % aqueous acetic acid. In this solvent two main spots were evident, R_F 0.70 strong and R_F 0.50 weak. The reaction forming β-conidendric acid, however, as would be expected from a study of its stereochemistry and lactonization rates of the dimethyl-β-conidendric acid[1], was more complicated. An attempt to produce β-conidendric acid using the same experimental conditions advocated by HOLMBERG[1] for α-conidendric acid resulted in a mixture of products heavily contaminated with the lactone. Paper chromatography of the mixture in 2 % acetic acid produced two main spots, R_F 0.77 medium and R_F 0.00 strong (β-conidendrin) with considerable streaking from the origin to R_F 0.58 as is found in papergrams of β-conidendrin in this solvent.

Because of the small quantities and labile nature of these reaction products of α- and β-conidendrin only α-conidendric acid can be assigned with certainty. The other compounds, however, must be closely related since they appear to be in equilibrium with the lactone and its hydroxy acid, although the possibility of some form of double spot chromatographic behaviour cannot be excluded.

TABLE I

CHROMATOGRAPHIC DATA OF α- AND β-CONIDENDRIN, CONIDENDRIC ACID AND SIMILAR LIGNANS

Lignan	*Solvent system**									
	2 % acetic acid			*Butanol–acetic acid–water (60:15:25, v/v)*			*Methanol–amyl alcohol–benzene–water (2:1:1:1, v/v)*			
	R_F value	*FF*	*DSA*	*R_F value*	*FF*	*DSA*	*R_F value*	*FF*	*DSA*	*Acid*
α-Conidendrin	↓ 0.00 S	Blue	Pinkish brown	0.83 S	Blue	Pinkish brown	0.81 S	Blue	Pinkish brown	
	0.50 M	Blue	N.D.							
	0.70 VW	Pale blue	N.D.							
α-Conidendrin–aqueous acetic acid (1:1) reaction mixture	↓ 0.00 S	Blue	Pinkish brown	0.86 S	Blue	Pinkish brown	0.75 W	Blue	Pink	Pale yellow
	0.50 M	Blue	Pinkish brown				0.81 S	Blue	Pinkish brown	
	0.70 W	Pale blue	Pink							
α-Conidendrin–aqueous acetic acid (1:1) reaction mixture after solvent removal	↓ 0.00 S	Blue	Pinkish brown	0.86 S	Blue	Pinkish brown	0.75 M	Blue	Pink	Pale yellow
	0.70 M	Blue	Pinkish brown				0.81 S	Blue	Pinkish brown	

β-Conidendrin	↓	0.00 S 0.58 W	Blue Blue	Pinkish brown N.D.	0.86 S	Blue	Pinkish brown	0.82 S	Blue	Pinkish brown
β-Conidendrin–aqueous acetic acid (1:1) reaction mixture (unchanged after solvent removal)	↓	0.00 S 0.58 W	Blue Blue	Pinkish brown N.D.	0.87 S	Blue	Pinkish brown	0.82 S	Blue	Pinkish brown
α-Conidendric acid		0.50 W 0.70 S	Blue Blue	Pinkish brown Pinkish brown	0.86 S	Blue	Pinkish brown	0.75 S	Blue	Pinkish brown
β-Conidendric acid heavily contaminated with β-conidendrin	↓	0.00 S 0.58 W 0.77 M	Blue Blue Blue	Pinkish brown Pinkish brown Pinkish brown	0.69–0.94 single elongated spot	Blue	Pinkish brown	0.64–0.90 single elongated spot	Blue	Pinkish brown
Matairesinol		0.60 S	Blue	Red purple	0.94 S	Blue	Red purple	0.92 S	Blue	Red purple
Hydroxymatairesinol		0.85 S	Blue	Orange	0.90 S	Blue	Orange	0.77 S 0.85 W**	Blue Blue	Orange Pinkish brown

Pale yellow	
N.D.	

* Detection: FF = ferric ferricyanide reagent[3]; DSA = diazotized sulfanilic acid reagent[2]; acid = bromocresol green plus bromophenol blue acid detector[4]; ↓ = streaking; S = strong; M = medium; W = weak; VW = very weak; N.D. = not detectable.

** α-Conidendrin[5].

The author wishes to thank Dr. H. L. HERGERT for the sample of hydroxymatairesinol and Crown Zellerbach, Chemical Products Division, Camas, Washington, U.S.A. for samples of α- and β-conidendrin.

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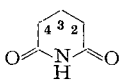
R_F values of some glutarimides

Various derivatives of glutarimide (I) have been shown to possess biological activities (Table I)[1,2] and a few of these are employed in medical practice. As part of an investigation of the metabolic fate of some glutarimides in animals, a study was made of their chromatographic properties.



(I)

All paper chromatograms were carried out with Whatman No. 1 grade. Some papers were impregnated with liquid paraffin (4% in hexane), olive oil (20% in

TABLE I

MAIN PHARMACOLOGICAL ACTION OF THE GLUTARIMIDES USED IN THE STUDY

	Compound	*Pharmacological action*
I	Glutarimide	None
II	3,3-Dimethyl glutarimide	Convulsive
III	3-Ethyl-3-methyl glutarimide (Bemegride)	Convulsive
IV	2,4-Dicyano-3-ethyl-3-methyl glutarimide	None
V	3-Isopropyl-3-methyl glutarimide	Convulsive
VI	3,3-Di-*n*-propyl glutarimide	Hypnotic
VII	2-Phenyl-2-ethyl glutarimide (Glutethimide)	Hypnotic
VIII	2-*p*-Aminophenyl-2-ethyl glutarimide (Aminoglutethimide)	Anticonvulsive
IX	2-Phenyl-2-diethylaminoethyl glutarimide hydrochloride (Phenglutarimide)	Parasympatholytic

TABLE II

R_F VALUES (× 100) OF SOME GLUTARIMIDES ON CELLULOSE PAPER

Glutarimide	*Solvent systems**																		
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{12}**	S_{13}	S_{14}	S_{15}	S_{16}	S_{17}	S_{18}	S_{19}
I	70	62	83	74	68	77	50	68	66	56	92	68	78	67	60	57	20	100	31	100
II	84	72	84	84	—	75	84	74	90	84	—	89	24	80	84	—	100	98	74	96
III	100	71	83	86	81	86	84	74	91	85	100	86	100	84	85	85	100	100	90	76
IV	—	—	—	40	—	89	—	—	63	87	83	97	—	84	88	67	85	96	3	93
V	89	67	81	—	—	89	91	67	95	89	100	91	—	86	86	—	97	100	100	—
VI	91	—	60	100	91	93	100	80	96	—	100	97	—	88	97	90	100	98	100	63
VII	91	76	60	86	84	83	98	80	93	90	97	90	100	82	91	86	97	100	100	10
VIII	84	70	71	—	5	86	—	72	72	80	97	75	—	73	74	97	87	96	15	80
IX	64	68	65	56	71	86	68	72	64	56	—	—	78	61	57	53	11	98	5	94

* S_1 = *n*-propanol–water (4:1), 4 h; S_2 = methanol–petroleum ether 40–60° (1:1), 5 h; S_3 = *n*-hexanol–water (1:1), 7 h; S_4 = benzyl alcohol–water (1:1), 7 h; S_5 = methanol–chloroform (1:1), 5 h; S_6 = ethanol–0.9% (w/v) sodium chloride (1:1), 6 h; S_7 = ethanol–0.2 *N* HCl (1:1), 7 h; S_8 = methanol–pyridine–water (1:20:5), 4 h; S_9 = *n*-butanol–acetic acid–water (12:3:5), 7 h; S_{10} = *n*-butanol–ethanol–water (3:1:1), 10 h; S_{11} = *n*-butanol–dimethyl formamide–water (15:4:1), 8 h; S_{12} = isobutanol–acetic acid–water (4:1:1), 12 h; S_{13} = *tert.*-butanol–acetic acid–water (4:1:1), 24 h; S_{14} = *tert.*-butanol–acetic acid–water (4:1:1), 17 h; S_{15} = isopropanol–ethyl acetate–water (2:1:3), 4 h; S_{16} = toluene–acetic acid–water (10:5:4), 1 h; S_{17} = chloroform–acetic acid–water (1:2:1), 8 h; S_{18} = carbon tetrachloride–acetic acid–water (1:2:1), 7 h; S_{19} = sodium chloride 10% (w/v), 2 h.

** Cellulose phosphate paper.

acetone[3]) or tributyrin (10 % in acetone). All compounds were applied to the sheets in amounts of 100 μg by the window technique[4] and they were located with the hypochlorite reagent of GRIEG AND LEABACK[5]. Thin-layer chromatographic separation was made on alumina (Merck) plates, 50 μ thick, prepared with a Camag thin-layer chromatographic spreader and activated at 150° for 1 h. Alkaline solvent systems could not be used because the glutarimides, especially the 2-substituted derivatives, hydrolyse above pH 8. Ascending chromatography was used in all experiments and the data are tabulated in Tables II, III and IV.

By the use of any two of four solvent systems it was possible to distinguish between the glutarimides, the most useful being toluene–acetic acid–water (10:5:4), carbon tetrachloride–acetic acid–water (1:2:1), aqueous sodium chloride (10 %, w/v)

TABLE III

R_F VALUES (× 100) OF SOME GLUTARIMIDES ON IMPREGNATED CELLULOSE PAPER

Glutarimide	*Solvent systems* *				
	S_1 **	S_{20} ***	S_{21} ***	S_{22} §	S_{23} §
I	69	54	83	80	87
II	86	61	—	72	70
III	81	64	84	50	68
V	—	65	86	35	—
VI	—	66	79	5	3
VII	—	63	76	2	4
VIII	—	—	—	20	20
IX	65	68	88	83	87

* S_1 = *n*-propanol–water (4:1), 24 h; S_{20} = methanol–water (1:1), 8 h; S_{21} = acetic acid–water (1:1), 8 h; S_{22} = 0.066 *M* sodium phosphate pH 7.3, 1 h; S_{23} = 0.066 *M* sodium phosphate pH 8.0, 1 h.
** Paper impregnated with liquid paraffin.
*** Paper impregnated with olive oil.
§ Paper impregnated with tributyrin.

TABLE IV

R_F VALUES (× 100) OF SOME GLUTARIMIDES ON THIN-LAYER ALUMINA PLATES

Glutarimide	*Solvent systems* *							
	S_1	S_{11}	S_{14}	S_{22}	S_{24}	S_{25}	S_{26}	S_{26} **
I	80	87	87	98	69	—	—	—
II	85	89	95	92	70	—	—	—
III	87	88	92, 74 ***	90	73, 65 ***	95	79	62
VII	86	94, 85 ***	87	93, 71 ***	70	95	82	68
VIII	85	88	86	90	70	—	—	—
IX	77	85	78	95	67	95	75, 11 ***	14

* S_1 = *n*-propanol–water (4:1), 2 h (time for 5 in. rise of solvent front); S_{11} = *n*-butanol–dimethyl formamide–water (15:4:1), 2.5 h; S_{14} = *tert.*-amyl alcohol–acetic acid–water (4:1:1), 10 h; S_{22} = 0.066 *M* sodium phosphate pH 7.3, 1 h; S_{24} = methanol, 0.75 h; S_{25} = chloroform–methanol (1:1), 0.5 h; S_{26} = chloroform–acetone (9:1), 1 h.
** Plate activated at 180° for 3 h.
*** Formation of double spots.

and, on paper impregnated with tributyrin, 0.066 *M* sodium phosphate, pH 7.3. With these solvents compact spots of reproducible R_F were obtained. The systems isobutanol–acetic acid–water (4:1:1) and *tert.*-butanol–acetic acid–water (4:1:1) gave good resolution but required longer times for development. *n*-Butanol–acetic acid–water (12:3:5) was also an efficient system and has been found to separate the metabolites of Bemegride[6].

Development of the alumina chromatoplates with chloroform or chloroform–benzene (1:1) resulted in the appearance of multiple spots with the compounds used. This was also observed with a number of other solvents for a few of the glutarimides (Table IV). All of the spots were well resolved and the compounds were easily detected in amounts of 1–10 μg by the hypochlorite reagent and by the alkaline hydroxylamine spray of SHEPPARD *et al.*[7]. This is particularly useful, because the 3-substituted glutarimides are not readily located by the latter reagent on paper chromatograms. The detection of glutethimide, in these small quantities, by the hydroxylamine reagent on alumina plates may be of importance in forensic investigations. Barbiturates, which may be present with the glutarimide in biological specimens taken for toxicological analysis, do not interfere with the reaction.

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DAWN DAVIES
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A sensitive semi-quantitative method for amino-acid analysis

Methods have been developed for the analysis of hydrolysates of peptides, available in only 0.01 to 0.1 μmole amounts, using high-voltage electrophoresis (HVE) on filter paper followed by quantitative reaction of the separated amino acids with ninhydrin[1,2]. The technique has the disadvantage that certain groups of amino acids cannot be resolved, and although improved resolution may be obtained by HVE at two different pHs the procedure requires double the amount of peptide and ambiguities still occur. This difficulty has been overcome to some extent by the use of two-dimensional systems employing ionophoresis in one direction followed by either ionophoresis at a different pH[3-5] or by paper chromatography[6], in a direction at right angles. Such procedures suffer in comparison with the single-dimensional techniques in that they are less sensitive and more time-consuming. This paper describes a small-scale two-dimensional method that allows simultaneous fractionation of ten peptide hydrolysates in 3 h. Subsequent reaction with ninhydrin provides semi-quantitative analysis requiring only 2 to 20 mμmoles of peptide.

Experimental

A piece, 114 cm long, was cut from a roll of Whatman No. 3 MM filter paper 6 in. wide, and placed on a clean sheet of polythene 48 in. long by 8 in. wide. The paper was marked symmetrically with six pencilled lines at right angles to its length to delineate five rectangles 7 in. by 6 in. Five points were marked, 1.5 cm from one long edge of the paper and 4.5 cm from the right-hand (anode) edge of each rectangle, with the identifications of the hydrolysates to be applied alongside them. The paper was then impregnated, as described by ATFIELD AND MORRIS[2], with an aqueous buffer at pH 1.85 containing 74.25 ml of glacial acetic acid and 21.0 ml of 90% formic acid per litre. The five hydrolysates, prepared from between 2 and 20 mμmoles of peptide, were applied in volumes less than 1 μl to provide spots sufficiently compact for good resolution. HVE was carried out for 7 min at 20 kV (182 V/cm) and about 100 mA. The HVE apparatus was fitted with water-cooled plates 42 in. long and $6^1/_4$ in. wide, the emergent temperature of the cooling water being 11°. After ionophoresis the paper was dried in a current of air at 40° and cut along the six pencilled lines. The five rectangular pieces of paper so formed were each punched with two $^1/_4$ in. holes set 3 in. apart and $^1/_2$ in. from the edge of the long side opposite the point of application of the hydrolysate, and together with another five sheets previously prepared, assembled on two glass rods on the stainless-steel apparatus shown in Fig. 1.

This apparatus was mounted in a cubic glass tank of side 1 ft., with the two actuating rods passing through two holes in the lid, which were sealed with two corks when the lid was in position. The organic phase of one of the mixtures of solvents shown in Table I was contained in the rectangular glass dish that fitted onto the adjustable metal stands: a second dish containing the aqueous phase was placed beneath the stand on the bottom of the tank. After an appropriate interval for equilibration, during which the two actuating rods were raised to their fullest extent and the bottom edges of the papers about $^1/_2$ in. above the level of the solvent, the actuating rods were depressed until the papers just dipped into the solvent. Development was allowed to proceed until the solvent front reached the top edges of the papers,

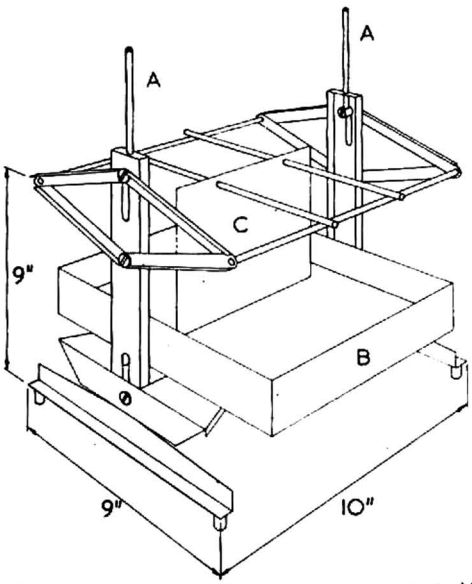


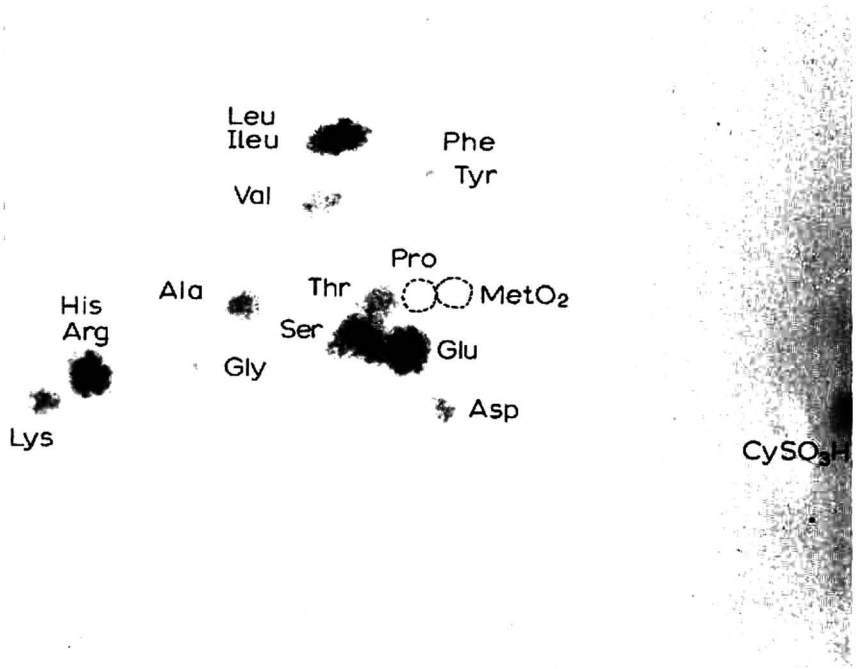
Fig. 1. Stainless-steel chromatogram support. (A) Actuating rods; (B) dish for mobile phase; (C) chromatogram.

usually about $2^1/_2$ h. The papers were then removed, dried in warm air, treated with cadmium–ninhydrin reagent[2,7] and stored overnight in an ammonia-free atmosphere for the colours to develop.

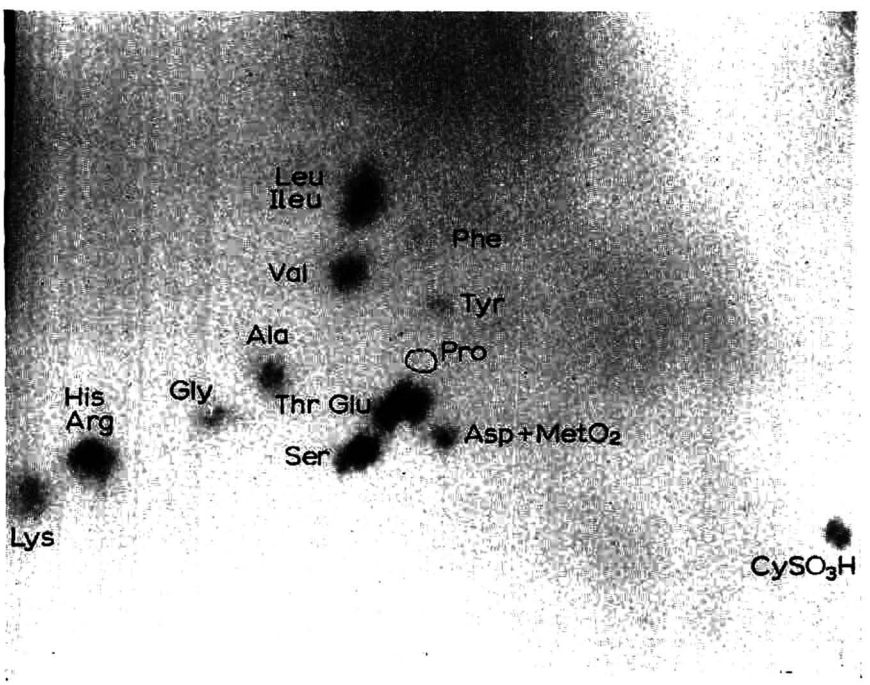
Quantitative measurements of the amounts of amino acids separated were made by cutting out the spots, allowing each of them to stand for 30 min with 1.0 ml of methanol in a small polythene-capped glass vial, and measuring the red colour extracted in micro-cells of 2 cm path-length at 500 mμ (260 mμ in the case of proline). After deduction of the reading given by an equal area of unstained paper, amounts of amino acids were obtained by reference to data obtained with known amounts of controls.

Results

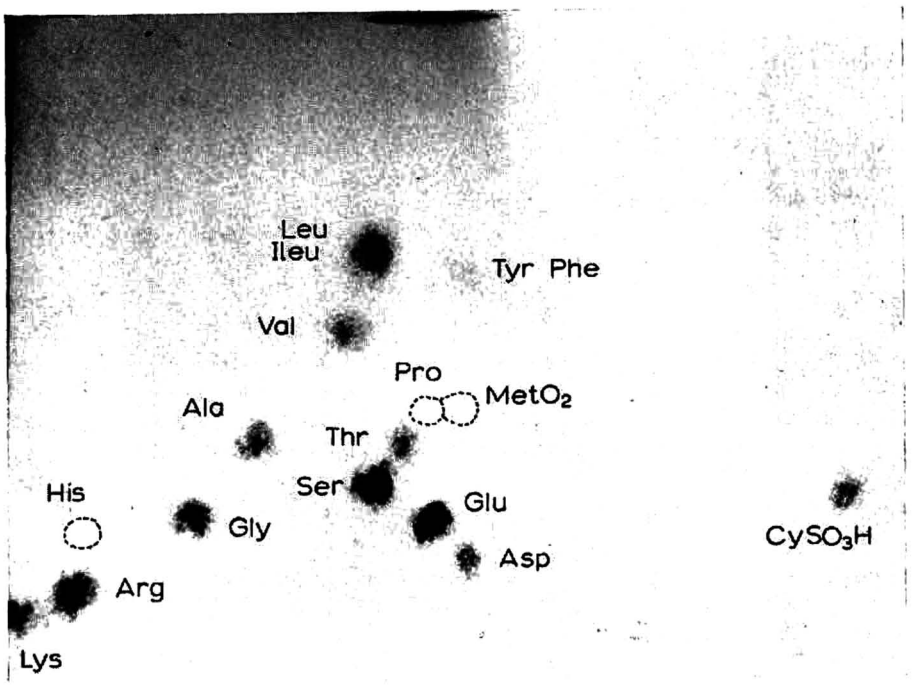
No developing solvent could be found that would fully resolve every amino acid normally found in hydrolysates of oxidised proteins. Table I lists the solvent systems that have been found useful and notes those pairs of amino acids that are not separated. The solvent system D requires a full day for chromatography. A double development with *sec.*-butanol–3 % aqueous NH_3 (5:2, v/v) will separate all amino acids, including isoleucine and leucine, in 7 h, but is unsuitable for quantitative measurements because of the background contamination with residual ammonia. Fig. 2 shows reproductions of actual separations obtained in representative experiments. The position of proline is marked with a dotted line as it is not clearly visible in the photographs. Cystine and methionine have not been included in the investigation because they do not occur as such in oxidised proteins, but according to their known electrophoretic mobilities and R_F values they should be completely resolved in most of the solvent systems quoted. Table II shows the results obtained in the


Leu
Ileu
Phe
Tyr
Val
Pro
Ala
Thr
MetO2
His
Arg
Ser
Glu
Gly
Asp
Lys
CySO3H

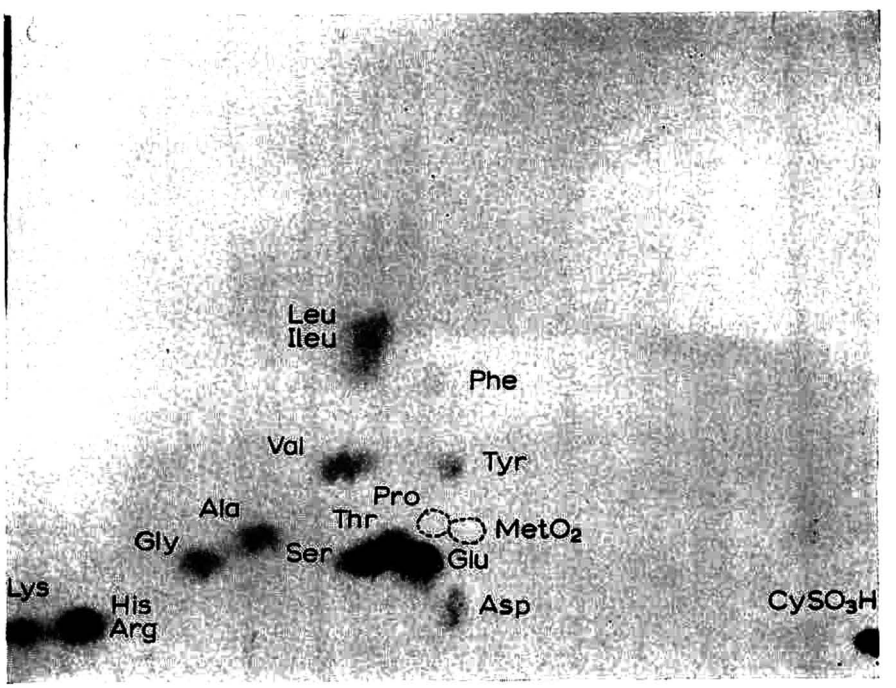
(a)


Leu
Ileu
Phe
Val
Tyr
Ala
Pro
His
Arg
Gly
Thr Glu
Asp+MetO2
Ser
Lys
CySO3H

(b)



(c)



(d)

Fig. 2. Chromatograms obtained after development with the solvents given in Table I. (a) Solvent A; (b) solvent B; (c) solvent C; (d) solvent D.

TABLE I

SOLVENTS FOR PARTITION CHROMATOGRAPHY

Solvent system	*Amino acids not separated*
(A)	
n-Butanol–pyridine–acetic acid–water (15:10:3:12)	Histidine–arginine Leucine–isoleucine
(B)	
n-Butanol–acetic acid–water (4:1:5)	Methionine sulphone–aspartic acid Leucine–isoleucine partially resolved Histidine–arginine partially resolved Threonine–glutamic acid partially resolved
(C)	
2,4/2,5-Lutidine–ethanol–water (11:4:5)	Leucine–isoleucine Tyrosine–phenylalanine partially resolved
(D)	
tert.-Amyl alcohol saturated with water. Double development	Histidine–arginine

TABLE II

SEPARATION AND ESTIMATION OF AN EQUIMOLECULAR MIXTURE OF LEUCINE, ASPARTIC ACID, GLYCINE AND LYSINE

Amino acid applied ($m\mu$ *mole*)	*Leucine*		*Aspartic acid*		*Glycine*		*Lysine*	
	Found ($m\mu$ *mole*)	% *Recovery*	*Found* ($m\mu$ *mole*)	% *Recovery*	*Found* ($m\mu$ *mole*)	% *Recovery*	*Found* ($m\mu$ *mole*)	% *Recovery*
1.44	1.26	88	1.43	100	1.84	128	1.36	95
2.84	3.03	107	3.44	122	4.06	143	2.69	95
4.23	4.73	112	3.79	89	4.12	98	4.07	96
5.70	5.45	96	5.89	103	5.79	101	5.85	103
6.70	6.74	101	6.51	97	6.19	92	7.53	112

analysis of an equimolecular mixture of leucine, aspartic acid, glycine and lysine. The greatest error would be expected to occur in the determination of glycine since this amino acid gives by far the lowest colour yield with the cadmium-ninhydrin reagent.

Discussion

The method has been found extremely useful for the elucidation of the structures of peptides isolated from the tryptic digest of a protein fraction obtained from oxidised wool. The complexity of this tryptic digest was such that pure peptides were only obtained after a final purification by paper chromatography or paper ionophoresis, and the amounts of pure peptides available were consequently small (< 0.3 μmole). The method is most suitable for the analysis of the products of partial hydrolysis of such a peptide, since a solvent system can usually be found that will separate all its constituent amino acids. The sensitivity and accuracy are good

enough to permit unequivocal determination of the amino-acid composition of small peptides.

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Chromatography on ion exchange papers

XVI. The adsorption of metal ions on cation exchangers from solutions of sodium perchlorate

The adsorption of metal ions from $HClO_4$ on sulphonic cation exchangers was discussed by NELSON *et al.*[1] and by LEDERER AND SARACINO[2]. No adequate explanation for the increase in adsorption of metal ions in higher concentrations of $HClO_4$ has so far been advanced. NELSON *et al.*[1] pointed out that accurate information for a number of the variables, *e.g.*, electrolyte invasion of the resin, activity coefficients of the metal ions in the supporting electrolyte, was lacking. We have shown[2] that this phenomenon was not confined to sulphonic polystyrene resins but occurred also with cellulose sulphonic exchangers. As no data were available for the behaviour of metal ions in various perchlorates as electrolytes we decided to investigate perchlorates in the hope that the data might shed some light on the problem.

Chromatography was carried out on Amberlite SA-2 resin paper as described previously[2]. The perchlorate solutions used as developing solvent had to contain 1 *N* $HClO_4$ so as to avoid hydrolysis of the metal ions and to avoid the formation of several fronts by demixion during development. Amongst the salts of perchloric acid only the sodium and the barium salt are sufficiently soluble in water to permit comparisons with $HClO_4$ over a wider range of concentrations. Preliminary results with barium perchlorate showed that it changed the equilibria owing to the divalent cation and hence only sodium perchlorate (containing 1 *N* $HClO_4$) could be compared

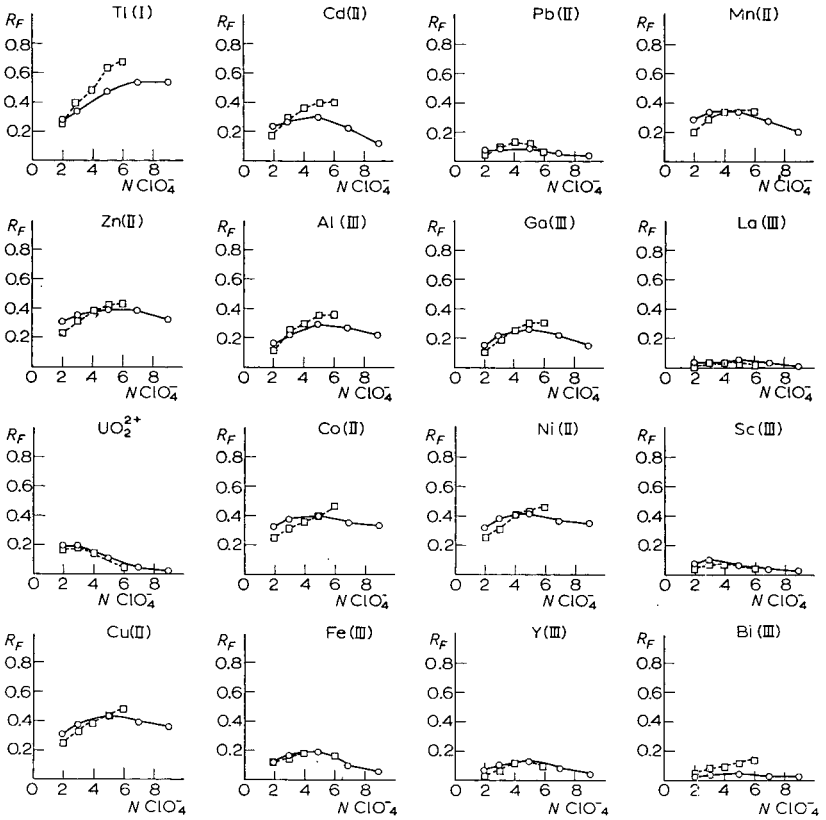


Fig. 1. R_F values of metal ions on sulphonic resin paper Amberlite SA-2 plotted against the concentration of ClO_4^-. ○—○—○ = the sodium form of the paper developed with mixtures of $NaClO_4$ with 1 N $HClO_4$. □—□—□ = the hydrogen form of the paper developed with $HClO_4$.

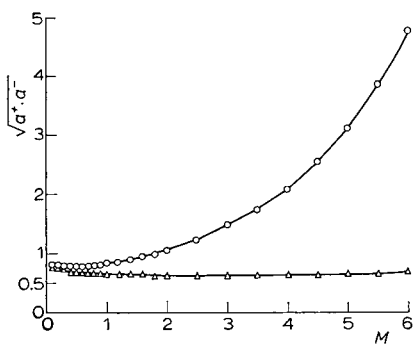
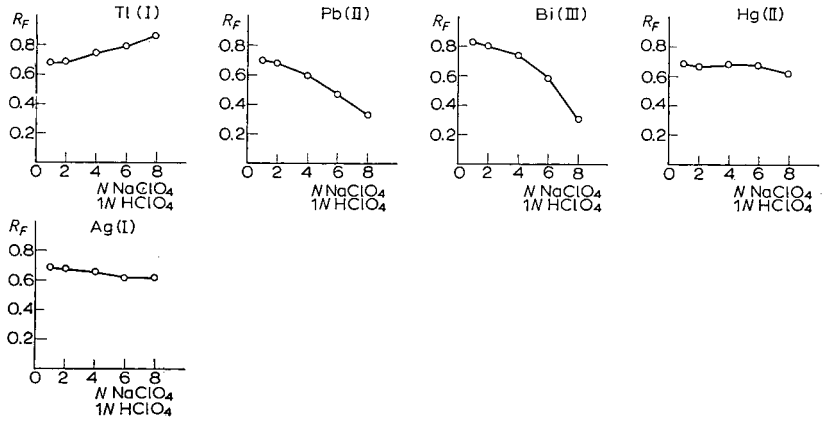
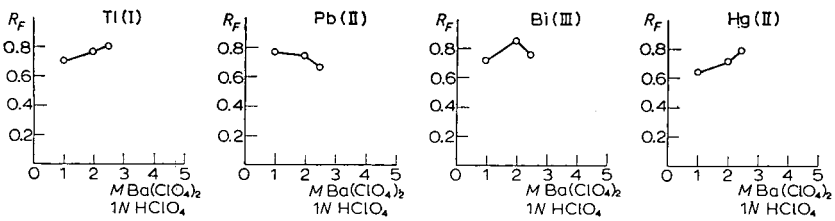


Fig. 2. The activity of perchloric acid and sodium perchlorate plotted against the concentration (values taken from ref. 3). ⊙——○ = $HClO_4$; △—△ = $NaClO_4$.



(a)



(b)

Fig. 3. R_F values of some metal ions on Whatman No. 3MM (cellulose) paper developed (a) with mixtures of $NaClO_4$ and 1 N $HClO_4$ plotted against the concentration of $NaClO_4$, and (b) with mixtures of $Ba(ClO_4)_2$ and 1 N $HClO_4$ plotted against the concentration of $Ba(ClO_4)_2$.

with $HClO_4$. Fig. 1 shows the R_F values of 16 metal ions both in sodium perchlorate and perchloric acid and we were surprised to find that for all intents and purposes the two electrolytes give the same R_F values for all ions. This is the more surprising as the activity coefficients of $HClO_4$ and $NaClO_4$ differ considerably at higher concentrations as shown in Fig. 2.

Fig. 3 shows the R_F values of a number of ions on cellulose paper with $NaClO_4$ and $Ba(ClO_4)_2$ as solvents. The ions examined here had shown adsorption in $HClO_4$[2].

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1 F. NELSON, T. MURASE AND K. A. KRAUS, *J. Chromatog.*, 13 (1964) 503.
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Announcements

Colloquium on Protides of the Biological Fluids

The XIIIth Annual Colloquium on Protides of the Biological Fluids will be held in Bruges, Belgium, on 29th and 30th April and 1st and 2nd May, 1965.

PROGRAMME

Topics

I. Lipoproteins
II. Proteins of the nervous system
III. Impedance measurements on proteins.

Round Table Discussions:

I. The physical, chemical and clinical method of lipoprotein analysis
II. The proteins of the nervous system.

For application and communications, please apply to:
Colloquium on Protides of the Biological Fluids, P.O. Box 71, Bruges (Belgium).

J. Chromatog., 17 (1965) 428

Assembly of the Swiss Society of Clinical Chemistry

The Swiss Society of Clinical Chemistry will hold its next assembly in Basle on May 22nd–23rd, 1965. The scientific part will be devoted to chromatography, including paper, thin-layer, column and gas chromatography.

Information can be obtained from Dr. T. Brechbuhler, Kinderspital Basle, Switzerland.

J. Chromatog., 17 (1965) 428

3rd International Symposium on Advances in Gas Chromatography

The Third International Symposium on Advances in Gas Chromatography will be held at the Sheraton-Lincoln Hotel in Houston, Texas, October 18–21, 1965. An outstanding program is being planned which will include lectures by internationally known leaders in this field. New developments in detectors and columns for liquid chromatography will be included in the program. There will also be an exhibition of new gas chromatographic instruments and accessories.

Participation in the Symposium will be on the basis of invited contributions; however, a limited number of papers from the U.S. and abroad are being sollicited. Those who wish to make contributions are requested to submit an abstract of about 700 words (or the complete manuscript) by June 1st.

Financial assistance for travel will be available for authors of papers accepted from abroad.

Abstracts of papers and inquiries concerning the conference should be directed to: Prof. A. Zlatkis, Department of Chemistry, University of Houston, Houston, Texas, U.S.A.

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Thin-layer Chromatography

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See PC section.

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Gas Chromatography

1. REVIEWS AND BOOKS

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2. FUNDAMENTALS, THEORY AND GENERAL

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2b. Gas–solid systems

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2c. Thermodynamics and theoretical relationships

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GORDON, S. M., KRIGE, G. J. AND PRETORIUS, V.: The theory of preparative linear chromatography: The role of the flow velocity. *J. Gas Chromatog.*, 2 (1964) 241–245 — the efficiency increases with the flow velocity and the maximum is broader than the optimum flow velocity of analytical columns.

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MARTIRE, D. E., PECSOK, R. L. AND PURNELL, J. H.: Liquid–gas interface effects in gas chromatography. *Nature*, 203 (1964) 1279–1280 — no significant error is introduced in ignoring a Gibb's surface correction when columns have more than 5% liquid loading and the activity coefficient is less than 10.

2d. General

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3. TECHNIQUES I

3a. Detectors

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FOWLIS, I. A., MAGGS, R. J. AND SCOTT, R. P. W.: The critical examination of commercially available detectors for use in gas chromatography. Part I. The macro argon and flame ionisation detectors. *J. Chromatog.*, 15 (1964) 471–481 — flame-ionisation detection was found to be linear in the concn. range 10^{-6}–10^{-10} g/ml; macro argon detection is linear over a small concn. range at one particular voltage; sensitivities 10^{-11} and 10^{-13} g/ml, resp.

ROESLER, J. F.: Preliminary study of characteristics of photoionization detector for gas chromatography. *Anal. Chem.*, 36 (1964) 1900–1903 — *ca.* 5 ml N_2 sample with 2.8 p.p.m. of C_3H_8 gives a maximum response of 660 $\mu\mu$A at a noise level of 70 $\mu\mu$A.

TILLEY, J. M. A., CANAWAY, R. J. AND TERRY, R. A.: The estimation of volatile fatty acids by gas chromatography with automatic titration. *Analyst*, 89 (1964) 363–365 — circuit of the photoelectric detector control unit is given.

3b. Column performance and filling studies

COSTA NETO, C., KÖFFER, J. T. AND DE ALENCAR, J. W.: Programmed flow gas chromatography. Part I. *J. Chromatog.*, 15 (1964) 301–313 — GC separation at different carrier gas velocities, which are programmed discontinuously.

DOBYTSHIN, D. P., PORSHNEVA, N. N. AND TURKEL'TAUB, N. M.: (Use of porous glasses for gas–solid and gas–liquid chromatography). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatographiya*, Izd. Akad. Nauk SSSR, Moscow, 1964, pp. 69–83 — the effect of pore diameter on the form of the isotherm is discussed in the case of permanent and hydrocarbon gases.

WACHSMUNDZKI, A., SUPRYNOWICZ, Z. AND PIETRUSIŃSKA, T.: (Investigation of the application of Carpathian diatomites as supporting materials in gas–liquid partition chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 721–729 — porosity and surface areas are given; usefulness comparable to that of Celite only for certain groups of compounds.

ZHDANOV, S. P., KISELEV, A. V., KALMANOVSKIĬ, V. I., FIKS, M. M. AND YASHIN, YA. I.: (Use of porous glasses as adsorbents for gas chromatography). A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatographiya*, Izd. Akad. Nauk SSSR, Moscow, 1964, pp. 61–68 — very good results with gaseous hydrocarbons.

3c. Apparatus, accessories and materials for GC

BARTZ, A. M. AND RUHL, H. D.: Rapid scanning infrared–gas chromatography instrument. *Anal. Chem.*, 36 (1964) 1892–1896 — two ranges, 2.5–7 μ and 6.5–16 μ, are covered in 16 sec by two single-beam grating spectrometers in parallel.

CROSBY, N. T. AND LAWS, E. Q.: The use of infrared spectroscopy in the analysis of pesticides residue. *Analyst*, 89 (1964) 319–327 — design of a simple semi-preparative gas chromatograph is given.

DEWAR, R. A. AND MAIER, V. E.: The flame ionization detector. A simple electrometer for linear, logarithmic and integral responses. *J. Chromatog.*, 15 (1964) 461–470 — a detailed description is given of a DC electrometer.

DUTTA, J.: A carrier-gas by-pass system for multiple gas–liquid partition chromatography apparatus. *Sci. Cult. (Calcutta)*, 29 (1963) 153–154 — 3 three-way and 2 four-way stopcocks enable any operation with three columns.

ERDMAN, K. L., MCDONALD, J. R., BEER, G. A. AND AXEN, D. A.: Simple gas circulation pump. *Rev. Sci. Instr.*, 35 (1964) 100–101 — useful for feeding of GC apparatus or for circulating techniques.

IBRAHIM, W. Y.: Note on stream-heated aluminium block for solvent evaporation of chromatographic fractions in presence of nitrogen. *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 390.

KARRMAN, K. J. AND HARALDSON, L.: (Simple laboratory gas chromatograph). *Elementa (Stockholm)*, 46 (1963) 301–306.

KONEČNÝ, K. AND STREIBL, M.: (Modification of a sampling device for the gas chromatograph "Chrom.-1"). *Chem. Listy*, 58 (1964) 1102–1105 — complete designs are given; used for C_{30} hydrocarbons.

LEVY, A.: The accuracy of the bubble meter method for gas flow measurements. *J. Sci. Instr.*, 41 (1964) 449–453 — errors ± 1% for flow rates of 0.1–1000 ml/sec may be reduced to $\pm$ 0.25% for flow rates of 0.1–100 ml/sec.

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WENDENBURG, J. AND JURISCHKA, K.: Eine einfache Vorrichtung zum Aufbrechen von abgeschmolzenen Glassampullen im Gaschromatographen. *J. Chromatog.*, 15 (1964) 538–540 — design is given.

ZHUKHOVITSKIĬ, A. A., TURKEL'TAUB, N. M. AND SHLYAKHOV, A. F.: (Preparation of dilute gaseous mixtures for chromatographic investigations). *Neftekhim.*, 4 (1964) 645–649 — a diffusion method is described for mixtures in which the concentration of the components is 0.1–1 p.p.m.

3d. Special methods

MASIKO, Y. I., KONOSU, H. AND MORII, T.: (Rapid elemental analysis by gas chromatography). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 67 (1964) 555–558; English summary A 37.

4. TECHNIQUES II

4a. Preparative-scale GC

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VERZELE, M.: The choice of carrier gas in preparative gas chromatography. *J. Chromatog.*, 15 (1964) 482–487 — the use of He and H_2 instead of N_2 is advocated for preparative GC

4d. Special microtechniques

REVEL'SKIĬ, I. A., BORODULINA, R. I. AND KHOKHLOVA, T. D.: (Continuous determination of H/C ratio in molecules of hydrocarbon mixtures and other organic mixtures). *Neftekhim.*, 4 (1964) 624–632 — after destruction to CO_2 and H_2 the H/C ratios are obtained with errors of about 3–5 % rel.

4e. Automation

DATSKEVICH, A. A., ZHUKHOVITSKIĬ, A. A. AND TURKEL'TAUB, N. M.: (Some questions of theory and practice of chromatography). In A. A. ZHUKHOVITSKIĬ *et al.* (Editors): *Gazovaya Khromatographiya*, Izd. Akad. Nauk SSSR, Moscow, 1964, pp. 44–49 — the relationship between zone spreading, column heating and concentration effect is discussed.

GITTINGS, R.: The application of process chromatography in industry. *Instr. Pract.*, 18 (1964) 235–238.

THOMPSON, W. R.: Conditioning on-line vapor fraction analyzer signals for process control. *ISA (Instr. Soc. Am.)*, 11, No. 6 (1964) 67–68.

4f. Measurement of physico-chemical and related values

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GAS CHROMATOGRAPHY OF VOLATILE AMINO ACID DERIVATIVES

II. LEUCINE, CYSTEINE, PROLINE, HYDROXYPROLINE, METHIONINE, PHENYLALANINE, ASPARTIC ACID AND GLUTAMIC ACID

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INTRODUCTION

In the previous paper[1] we reported on a variety of stationary phases which were screened for their possible application in the separation of the trifluoroacetyl (TFA) amino acid *n*-amyl esters of the seven most volatile amino acids. The present communication gives a similar set of data for the next seven amino acids, using the same volatile derivatives. For the reasons stated in the previous paper the separations were again carried out under isothermal conditions, but the number of phases screened is smaller, because some of those tried in the previous survey were not sufficiently stable at the higher temperatures required for these compounds.

MATERIALS AND METHODS

Apparatus

A D6 chromatograph (Griffin and George Ltd., Alperton, Middlesex) with a Martin gas-density balance detector was used. Columns consisted of two stainless steel tubes of 5 mm internal diameter connected at their lower end by a stainless steel capillary U-tube. Packed length was 182 cm. Nitrogen (99.9% "White spot", British Oxygen Co., Wembley, Middlesex) was used as carrier gas.

Preparation of columns

Silocel C22 firebrick (L. Light & Co. Ltd., Colnbrook, Bucks.) was crushed, graded and deactivated, coated with stationary phase and packed as described previously[1].

Stationary phases

The following stationary phases were obtained from F & M Scientific Europa N.V., Leidsestraat 67, Amsterdam: F-50 (methyl chlorophenyl silicone), SE-52 (phenyl methyl silicone elastomer), SE-54 (phenyl vinyl methyl silicone elastomer), XE-60 (cyanoethyl silicone elastomer), BDS (1,4-butanediol succinic acid polyester), castorwax, celanese ester No. 9 (tripelargonate) and Versamid 900 (polyamide from dimerised linoleic acid and ethylene diamine). MS type silicones were obtained from

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Hopkin & Williams, Chadwell Heath, Essex. The following phases were obtained from Applied Science Laboratories Inc., State College, Pa., U.S.A. through the courtesy of Dr. F. A. VANDENHEUVEL of the Canada Department of Agriculture, Ottawa: Hi-Eff-8B (cyclohexanedimethanol succinic acid polyester), ECN:SS-S and EGCN: SS-S (ethylene glycol succinate–cyanoethyl silicone copolymers), and EGSS-X (ethylene glycol succinate–methyl silicone copolymer). DEGA (diethylene glycol adipate), DEGS (diethylene glycol succinate), PEG-S (polyethylene glycol succinate) and PPS (polypropylene succinate) were obtained from Griffin & George Ltd., M & B (dimethyl polysiloxane) was obtained from May & Baker Ltd., Dagenham, Essex, QF-1 (fluorosilicone fluids FS 1265, 10,000 cs) from Midland Silicones Ltd., Barry, Glamorgan, SE 30 (dimethyl silicone elastomer) from I.C.I. Ltd., Stevenston, Ayrshire, PEG-A (polyethylene glycol adipate) from W.G. Pye & Co., P.O. Box 60, Cambridge, DSPO (Duo Seal Pump Oil) from T.J. Sas & Son Ltd., Vernon Place, Holborn, W.C.1., PEG 20M (polyethylene glycol) from Union Carbide Ltd., Grafton St., London, W.1., and PEG-L (polyethylene glycol lauryl ether) from Honeywill & Stein Ltd., Mayfair Place, London, W.1. We are indebted to Advita Ltd., Wellington Avenue, Walton-on-Thames, for generous gifts of Admul 19 (polyglycerol ester of mixed fatty acids) and Admul S57 (polyglycerol ester of hardened tallow fatty acids).

The method of FARQUHAR *et al.*[2] was used to prepare the following polyesters: BDA (1,4-butanediol adipate), NPGS (*neo*-pentyl glycol succinate), NPGG (*neo*-pentyl glycol glutarate), NPG Seb (*neo*-pentyl glycol sebacate), and EAA (ethanolamine adipate).

Preparation of derivatives

The N-TFA amino acid *n*-amyl esters were made by the method described previously[1]. With cysteine the N,S-bis TFA and with hydroxyproline the O,N-bis TFA derivatives were obtained. With aspartic and glutamic acids the di-*n*-amyl esters were formed.

RESULTS AND DISCUSSION

In Tables I, II and III retention times relative to aspartic acid are given for seven amino acids and bicumyl, which is a suitable standard for this group.

Catalytic decomposition effects were noted for some of the phases: the derivatives affected were those of cysteine and of hydroxyproline. Usually the phases concerned had been found to have a catalytic effect on the decomposition of the threonine and serine derivatives. The stability of the N-TFA, O-TFA and S-TFA derivatives is being investigated more fully.

On most phases tested the order of emergence of the derivatives is proline, methionine, phenylalanine, aspartic acid and glutamic acid. However, the order of emergence of the cysteine, hydroxyproline and proline peaks shows considerable variation, although these three always appear before the methionine peak. As previously noted[1], XE-60, which is regarded as more polar than the other silicone type phases, shows a closer resemblance to the phases in Table II than to those in Table I. EAA (Table III) is noteworthy because aspartic acid precedes methionine as well as phenylalanine in the order of emergence from the column.

There is a remarkable difference between the retention times of aspartic and

TABLE I

RELATIVE RETENTION DATA FOR SILICONE TYPE STATIONARY PHASES

The figures are the retention times relative to the aspartic acid derivative taken as 1.00. The actual retention time in min for this derivative is given in brackets. All packings were prepared with 5% w/w of liquid phase to support. Gas flow: 38 ml N_2/min; column temperature: 190°.

Liquid phase	*N-TFA, n-amyl ester*	*N,S-bis TFA, n-amyl ester*	*N,O-bis TFA, n-amyl ester*	*N-TFA, n-amyl ester*			*N-TFA, di-n-amyl ester*		*Bi-cumyl*	*HETP (mm) Asp peak*
	Leu	*CySH*	*Hypro*	*Pro*	*Met*	*Phe*	*Asp*	*Glu*		
MS 115	0.24	0.29	0.38	0.35	0.54	0.78	(25.8)	1.65	0.88	0.8
MS 200	0.22	0.28	0.36	0.34	0.51	0.76	(19.6)	1.68	0.84	2.1
MS 550	0.19	0.22	0.34	0.35	0.49	0.72	(13.4)	1.69	0.81	1.3
MS 710	0.15	0.19	0.30	0.39	0.53	0.80	(28.3)	1.87	1.10	0.5
M & B	0.21	0.25	0.33	0.34	0.50	0.74	(12.8)	1.64	0.84	3.0
F-50	0.21	0.26	0.33	0.32	0.52	0.80	(23.0)	1.71	0.90	0.7
QF-1	0.22	0.36	0.63	0.43	0.57	0.68	(26.3)	1.83	0.26	1.0
SE-30	0.21	0.27	0.35	0.36	0.52	0.71	(23.0)	1.63	0.86	1.2
SE-52*	0.16	0.22	0.32	0.30	0.47	0.73	(32.4)	1.82	0.78	1.0
SE-54*	0.15	0.20	0.31	0.31	0.46	0.71	(42.8)	1.80	0.76	1.3
XE-60	0.17	0.40	0.52	0.34	0.73	0.82	(34.2)	1.97	0.29	1.0

* Column temperature: 170°.

TABLE II

RELATIVE RETENTION DATA FOR POLYESTER TYPE PHASES

The figures are the retention times relative to the aspartic acid derivative taken as 1.00. The actual retention time in min for this derivative is given in brackets. All packings were prepared with 5%w/w of liquid phase to support. Gas flow: 38 ml N_2/min; column temperature: 190°.

Liquid phase	*N-TFA, n-amyl ester*	*N,S-bis TFA, n-amyl ester*	*N,O-bis TFA, n-amyl ester*	*N-TFA, n-amyl ester*			*N-TFA, di-n-amyl ester*		*Bi-cumyl*	*HETP (mm) Asp peak*
	Leu	*CySH*	*Hypro*	*Pro*	*Met*	*Phe*	*Asp*	*Glu*		
BDA	0.14	0.35	0.37	0.29	0.78	1.02	(49.0)	2.45	0.54	0.9
BDS	0.15	0.36	0.40	0.31	0.73	0.93	(32.6)	1.99	0.48	0.9
Hi-Eff-8B	0.14	—	—	0.30	0.70	0.94	(44.0)	2.10	0.61	0.9
DEGA	0.19	—	—	0.62	0.89	1.16	(39.3)	2.01	0.52	0.7
DEGS	0.15	—	—	0.37	0.81	1.03	(25.6)	1.99	0.50	0.7
NPGS	0.15	0.38	0.44	0.26	0.66	0.83	(49.3)	2.03	0.37	1.3
NPGG	0.14	—	0.41	0.28	0.72	0.91	(67.0)	2.16	0.42	0.9
NPG Seb	0.15	0.32	0.34	0.26	0.65	0.85	(44.0)	2.09	0.55	0.8
PEG-A	0.14	0.34	0.40	0.31	0.75	0.97	(47.8)	2.06	0.49	1.0
PEG-S	0.13	—	—	0.30	0.62	0.84	(56.5)	1.90	0.51	0.7
PPS	0.14	—	—	0.25	0.69	0.84	(68.5)	2.02	0.53	1.6

— = compound applied but gave no peak.

TABLE III

RELATIVE RETENTION DATA FOR MISCELLANEOUS TYPES OF STATIONARY PHASES

The figures are the retention times relative to the aspartic acid derivative taken as 1.00. The actual retention time in min for this derivative is given in brackets. All packings were prepared with 5% w/w of liquid phase to support. Gas flow: 38 ml N_2/min; column temperature: 190°.

Liquid phase	*N-TFA, n-amyl ester*	*N,S-bis TFA, n-amyl ester*	*N,O-bis TFA, n-amyl ester*	*N-TFA, n-amyl ester*			*N-TFA, di-n-amyl ester*		*Bi-cumyl*	*HETP (mm) Asp peak*
	Leu	*CySH*	*Hypro*	*Pro*	*Met*	*Phe*	*Asp*	*Glu*		
Admul 19	0.14	—	—	0.27	0.53	0.80	(55.5)	2.00	0.72	1.5
Admul S57	0.14	—	—	0.27	0.55	0.78	(50.0)	2.02	0.77	1.0
Castorwax	0.15	0.24	0.27	0.26	0.54	0.79	(55.2)	2.00	0.85	0.9
Celanese ester	0.14	0.28	0.31	0.22	0.54	0.77	(86.0)	2.07	0.60	1.1
DSPO	0.17	0.20	0.25	0.29	0.48	0.78	(36.0)	1.49	1.51	1.1
EAA*	0.12	—	—	0.32	1.18	1.25	(19.0)	2.26	0.38	1.7
ECNSS-S*	0.12	—	—	0.31	0.83	0.99	(74.2)	2.26	0.31	0.8
EGCNSS-S*	0.12	—	—	0.30	0.81	0.96	(69.5)	2.27	0.30	1.2
EGSS-X*	0.12	—	—	0.32	0.83	1.08	(53.2)	2.23	0.46	1.7
EGS	0.15	0.24	0.28	0.25	0.52	0.79	(58.2)	1.97	0.89	0.5
PEG 20M	0.12	—	—	0.30	0.62	0.82	(39.5)	1.67	0.58	2.3
PEG-L	0.12	—	—	0.30	0.62	0.85	(57.0)	1.96	0.63	0.5
Versamid 900	0.17	—	—	0.30	0.73	0.90	(39.2)	2.12	0.63	1.5

* Column temperature: 170°.
— = compound applied but gave no peak.

glutamic acids in view of the fact that there is a difference of only $-CH_2-$ between the molecules of the derivatives. A similar difference occurs between valine and leucine[1]. On the other hand, the proline and hydroxyproline derivatives differ by an O-TFA group but show only slight differences in retention times.

The inclusion of values for the leucine derivative makes it possible to relate the sequence on any phase of the amino acids in this investigation with that obtained with the previous group of amino acid derivatives[1]. It is important that the last peak

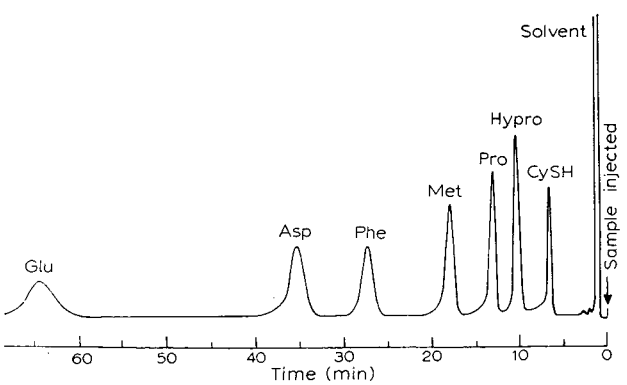

Fig. 1. Separation of a mixture of 7 trifluoroacetylated amino acid amyl esters, on Silocel C22 coated with 5% MS710 at 185°. Nitrogen flow rate: 38 ml/min.

of the first series will come off before the first peak of the second series. With every phase in Tables I, II and III the leucine peak emerges before that of any of the other seven amino acids; however, with DSPO (Table III) and some of the phases in Table I, some interference occurs as the leucine peak is close to the cysteine peak.

Of the phases investigated, only MS710 is capable of completely separating the seven derivatives (see Fig. 1). The next best phase is XE60, which, however, shows incomplete resolution of the methionine and phenylalanine peaks (Table I).

ACKNOWLEDGEMENTS

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SUMMARY

Data are presented for the relative retention times of the volatile derivatives prepared from leucine, cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid on 35 different stationary phases. The best resolution of these eight amino acids is obtained on Silicone MS710.

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ÄTHYLENGLYKOL-γ-KETOPIMELAT-POLYESTER ALS FLÜSSIGPHASE IN DER GAS–LIQUID-VERTEILUNGS-CHROMATOGRAPHIE

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Für gaschromatographische Trennungen mittel- und längerkettiger Aliphatengemische werden mit bestem Erfolg Polyester als polare Trennflüssigkeiten angewandt. Gegenüber unpolaren Verteilungsphasen haben sie den grossen Vorteil, dass unter analogen Bedingungen vor allem die Retentionszeiten von sauerstoffhaltigen Aliphaten wesentlich kürzer sind. Auf diese Weise lassen sich letztere bei tieferen Temperaturen trennen, ohne dass man eine Verlängerung der absoluten Retentionswerte und eine Verflachung der Elutionspeaks in Kauf nehmen muss.

Die auf dem Gebiet der Fettchemie hauptsächlich eingesetzten Polyester-Liquid-Phasen sind Polykondensationsprodukte zwischen aliphatischen Dicarbonsäuren mit einer Kohlenstoffkette von 4–10 C-Atomen und kurzkettigen mehrwertigen Alkoholen wie Äthylenglykol, Diäthylenglykol, Propan-1,3-diol und Butan-1,4-diol. Für Spezialzwecke kommen auch solche mit aromatischen Disäurekomponenten zum Einsatz, wie Äthylenglykol-isophtalat-polyester.

Die Polarität der Polyesterphasen ist abhängig vom Verhältnis der Anzahl der Methylengruppen zur Anzahl der Estergruppen im Kondensationsprodukt. Je mehr Estergruppen vorhanden sind, desto stärker wird die Polarität der Liquidphase. Die Trennung der gesättigten von kettengleichen ungesättigten Fettsäuremethylestern gelingt nach unserer Erfahrung mit bei $+20°$ flüssigem Äthylenglykol-succinat-polyester[1,*], als einer der polarsten rein aliphatischen Polyester-flüssigkeiten am besten. Gleiches gilt auch für die Analyse der Acetate kettengleicher gesättigter und ungesättigter Fettalkohole. Mit der flüssigen Succinat-polyesterphase lassen sich die Paare Stearinsäuremethylester–Ölsäuremethylester sowie Stearylacetat–Oleylacetat an 4 m langen gepackten Säulen gaschromatographisch völlig auftrennen. Dies gelingt aber mit den analogen schwächeren polaren Äthylenglykol-polyestern der Adipinsäure und Korksäure nicht mehr vollständig, und mit dem der Sebazinsäure ist der Trenneffekt noch weit ungünstiger.

An allen Polyesterphasen hingegen lassen sich die Homologen der schwächeren polaren aliphatischen sauerstoffhaltigen Verbindungen (Ester der Fettsäuren und der Fettalkohole) sehr glatt qualitativ und quantitativ analysieren. Die Trennung der Homologen der längerkettigen Alkohole in unverestertem Zustand untereinander und von kettengleichen ungesättigten ist aber selbst an stärkeren polaren Polyestersäulen recht unvollständig und die Peaks zeigen erhebliches "tailing", so dass die

* Wird erhalten durch Umesterung von Bernsteinsäuredimethylester mit überschüssigem Äthylenglykol in Gegenwart von Bernsteinsäure.

quantitative Auswertung mit recht grossen Fehlern behaftet bzw. vollkommen unmöglich ist.

HOFMANN UND STRUPPE[2] versuchten, die *n*-Alkohole mit 10 bis 20 C-Atomen an gepackten "Reoplex-400"-Säulen zu trennen. Nach den Angaben der Verfasser wurde keine befriedigende Auftrennung trotz Variierung aller gaschromatographischen Bedingungen erreicht. Die Retentionswerte waren sehr gross und die Peaks wurden sehr flach und unsymmetrisch erhalten.

Versuche von uns, Alkohole mit der stark polaren flüssigen Äthylenglykolsuccinat-polyesterphase zu trennen, scheiterten praktisch an den gleichen Schwierigkeiten. Die Retentionszeiten waren ebenfalls sehr lang und die Elutionskurven zeigten ähnlich starke "tailing".

ACKMAN UND SIPOS[3] analysierten die unveresterten kurzkettigen Carbonsäuren mit recht gutem Erfolg an gepackten Trennsäulen. Als Verteilungsflüssigkeit benutzten die Autoren das Polymerengemisch von Ketosebazinsäuren auf Chromosorb W bzw. auf dem inaktiven Träger Gas-Pack F.

Wir testeten zur gleichen Zeit Polyesterphasen, deren Säurekomponenten aus Ketodicarbonsäuren bestanden, auf ihre Trennwirkung für unveresterte aliphatische Alkoholgemische. Durch den Einbau der polaren Ketogruppe in den Polyester wurde die Trennleistung für Alkohole erheblich verbessert, die absoluten Retentionszeiten verkürzt und die Elutionspeaks in symmetrischen Kurven erhalten. Nach den Trennergebnissen mit Äthylenglykol-γ-ketopimelat-polyester als Verteilungsphase muss der Ketogruppe ein entscheidender Einfluss für die Auftrennung von Substanzen zugeschrieben werden, die ein leicht protonisierbares Wasserstoffatom an Sauerstoff gebunden enthalten und über Wasserstoffbrücken stark assoziierten.

EXPERIMENTELLES

1. Äthylenglykol-γ-ketopimelat-polyester

Ketodisäuren mit einer Kohlenstoffkette von weniger als 7 C-Atomen entzogen sich einer Polyveresterung mit Äthylenglykol. Sowohl bei der durch Säuren katalysierten Veresterung als auch bei neutralen Umesterungsreaktionen spalteten diese Dicarbonsäuren als α- bzw. β-Ketosäuren Kohlendioxid ab. Es resultierten hierbei niedrig molekulare und teilweise unübersichtliche Reaktionsprodukte. Die γ-Ketopimelinsäure hingegen liess sich sehr glatt mit Glykol verestern. Beide Komponenten reagierten miteinander in molaren Mengenverhältnissen unter Abspaltung von 2 Molen Wasser. Die Herstellung des Polyesters konnte praktisch auf die gleiche Art erfolgen wie sie von JAMES[4] für Succinat- und Adipat-polyester mitgeteilt wurde.

In einem 3-Halskolben mit Innenthermometer wird unter Reinststickstoffatmosphäre 1 Mol γ-Ketopimelinsäure[5] mit 1.05 Molen Äthylenglykol (wasserfrei, frisch destilliert) und 10 mg Toluolsulfonsäure innerhalb einer Stunde auf 180° erhitzt. Man lässt bei dieser Temperatur 2 St. weiterreagieren. Es destillieren hierbei etwa 36 g Wasser ab. Unter Anlegen von Wasserstrahlvakuum zieht man anschliessend den Überschuss an Glykol ab und steigert die Temperatur auf 200°. Nach weiteren 2 St. wird das Reaktionsgut bei angelegtem Vakuum und Schutzgaseinleiten erkalten gelassen.

Wir erhielten so in fast theoretischer Ausbeute einen schwach gelblich ge-

färbten Polyester als sirupöse Flüssigkeit, die nach mehreren Wochen Stehen bei einer Temperatur von 0° nicht erstarrte. Der Polyester war leicht löslich in Chloroform und Essigester. Mittels U.R.-Analyse konnte im Polyester die freie Ketogruppe nachgewiesen werden.

2. Apparatur

Zu allen gaschromatographischen Analysen wurden von uns zwei Eigenbaugeräte verwandt. Die Temperaturkonstanz der Luftthermostaten betrug bei Temperaturen oberhalb 200° ± 0.2°. Als Detektoren wurden Teilstrom-Wärmeleitfähigskeitsmesszellen benutzt, die im Institut für Verfahrenstechnik der Organischen Chemie der DAW zu Berlin entwickelt wurden[6] (industriell gefertigt von Firma Zimmermann, Leipzig). Sie waren mit den hängenden Trennsäulen aus Elektrolytkupfer (Durchmesser 6 mm) im senkrecht stehenden Thermostaten eingebaut. Das Dosiersystem liess sich über einen Regeltransformator bis zu 500° stufenlos beheizen. Es besass eine so grosse Wärmekapazität, dass eine momentane Verdampfung der Analysengemische gewährleistet war. Die Temperatur des Systems lag bei allen von uns durchgeführten Analysen etwa 50° über den Siedepunkten der Einzelsubstanzen bzw. denen der höchstsiedenden Komponenten bei Gemischen. Die Dosierung erfolgte mit einer Präzisionsdosierspritze, die bei festen Substanzen vorgeheizt war. Als Registriergerät verwendeten wir Kompensationsbandschreiber der Messgeräte- und Armaturenwerke "Karl Marx", Magdeburg-Buckau. Sie hatten einen Messbereich von 0–2.0 mV.

Die Trägermaterialien für die Flüssigphase bestanden aus Sterchamol (Merck) mit einer Korngrösse von 0.25–0.3 mm bzw. Celite C 29924 (Johns Mansville). 100 g Träger wurden jeweils mit 30 g des Ketopolyesters, der in Chloroform gelöst war, imprägniert und das Lösungsmittel im vorgeheizten und getrockneten Reinststickstoffstrom verdampft und anschliessend noch 5 St. lang unter weiterem Durchleiten des Inertgases auf 200° erhitzt und erkalten gelassen.

20 g vorstehend beschriebener Phase zeigten in einem Quarzrohr nach 20-stündigem Durchleiten bei 216° von 8 l Wasserstoffgas pro Stunde praktisch keinen Gewichtsverlust.

3. Trennung der Methylester von Fettsäuren

Für die Testung der Ketosäurepolyesterphase wurden von uns gaschromatographisch reine Methylester, die zum grössten Teil Syntheseprodukte darstellten, eingesetzt. Die Säulen wurden vor den quantitativen Messungen mehrmals mit den eingesetzten Estergemischen gesättigt. Fig. 1 zeigt die Trennung eines Modellgemisches der Methylester der gesättigten *n*-Fettsäuren mit einem Kettenlängenbereich von 9–20 Kohlenstoffatomen bei einer Temperatur von 216° an einer 4 m Säule. Die Retentionszeiten waren wesentlich kürzer als die bei vergleichbaren 4 m Reoplex-400- oder Succinatpolyestersäulen. Die Auftrennung in die einzelnen Komponenten entsprach etwa der der obigen Phase. Die quantitative Auswertung der Chromatogramme erfolgte planimetrisch und nach der Methode Peakhöhe × mittlere Peakbreite. Infolge der guten Symmetrie der Verteilungskurven ergaben beide quantitativen Methoden nach Tabelle I übereinstimmende Werte. In der gleichen Tabelle sind die Werte eines Modellgemisches der Methylester der geradzahligen *n*-Fettsäuren und der 3 ungesättigten C_{18}-Fettsäuren enthalten. Fig. 2 enthält das Chromato-

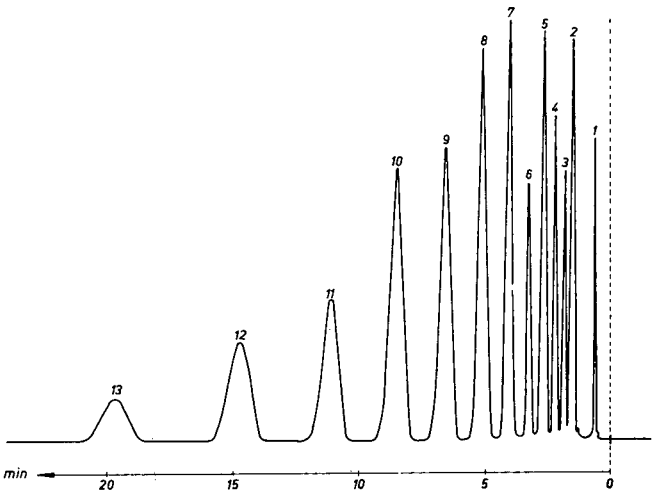

Fig. 1. Methylester der gesättigten Fettsäuren. Temperatur 216°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 9.1 l/Std. 1 = Diäthyläther; 2 = C_9; 3 = C_{10}; 4 = C_{11}; 5 = C_{12}; 6 = C_{13}; 7 = C_{14}; 8 = C_{15}; 9 = C_{16}; 10 = C_{17}; 11 = C_{18}; 12 = C_{19}; 13 = C_{20}.

gramm eines Gemisches der *n*-Fettsäuremethylester in einem Kettenbereich von C_8–C_{18} mit Ölsäure-, Linolsäure- und Linolensäuremethylestern. An der Ketopolyestersäule wurden Stearinsäure- und Ölsäureester ebenfalls wie an mittelpolaren Polyestersäulen nicht völlig aufgetrennt. Nach Fig. 3 verlief die Trennung der kürzeren gesättigten Fettsäureester von den kettengleichen Monoenfettsäureestern

TABELLE I

n-MONOCARBONSÄURE-METHYLESTER

Säure-methylester	*Ein-waage (Gew. %)*	*Gefunden Höhe × $^1/_2$ Peakbreite*		*Gefunden mit Planimeter*		*Ein-waage (Gew. %)*	*Gefunden Höhe × $^1/_2$ Peakbreite*		*Gefunden mit Planimeter*	
C_8	2.8	2.5	2.7	2.8	2.7	4.7	4.2	4.5	4.3	4.4
C_9	3.9	3.8	4.0	3.8	3.8					
C_{10}	5.2	5.4	5.4	5.3	5.4	8.0	8.1	7.9	7.9	8.0
C_{11}	5.7	5.8	5.5	5.9	5.6					
C_{12}	7.3	7.0	6.9	7.2	6.9	7.3	7.6	7.6	7.5	7.5
C_{13}	4.9	5.3	5.2	5.2	5.2					
C_{14}	10.2	10.6	10.6	10.6	10.5	8.6	9.0	8.6	8.8	8.5
C_{15}	8.6	8.7	8.9	8.6	8.8					
C_{16}	11.5	11.2	11.1	11.3	11.3	12.1	12.2	12.4	12.1	12.6
C_{17}	9.7	9.9	10.1	9.9	10.2					
C_{18}	10.9	10.6	10.5	10.5	10.6	13.5	14.5	14.3	14.6	14.2
C_{19}	8.6	8.8	8.4	8.7	8.4					
C_{20}	10.7	10.4	10.7	10.8	10.6					
$C_{18}{}^{=}$						18.2	17.1	17.0	17.6	17.2
$C_{18}{}^{==}$						16.9	17.1	17.0	17.6	17.2
$C_{18}{}^{===}$						10.7	9.6	10.3	9.7	10.2

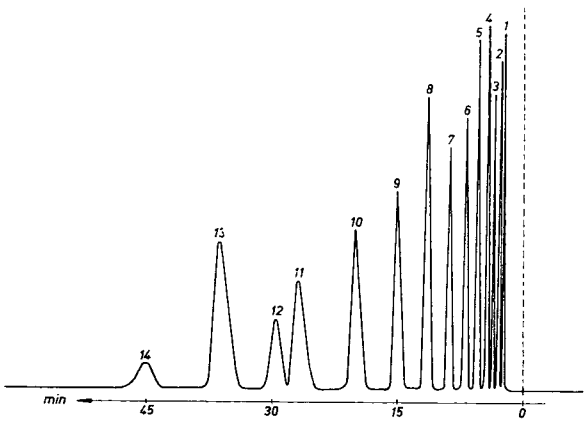

Fig. 2. Methylester der gesättigten Fettsäuren. Temperatur 200°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 6.2 l/Std. 1 = C_8; 2 = C_9; 3 = C_{10}; 4 = C_{11}; 5 = C_{12}; 6 = C_{13}; 7 = C_{14}; 8 = C_{15}; 9 = C_{16}; 10 = C_{17}; 11 = C_{18}; 12 = Ölsäuremethylester; 13 = Linolsäuremethylester; 14 = Linolensäuremethylester.

infolge der grösseren Polaritätsunterschiede weit günstiger. Die quantitative Auswertung nach Tabelle II ergab recht befriedigende Ergebnisse.

Nach den günstigen Trennergebnissen mit unveresterten Alkoholen, die später beschrieben werden, beschickten wir die Trennsäule mit Rizinolsäuremethylester mit unveresterter Hydroxylgruppe und anschliessend mit dem Hydrierungsprodukt obigen Esters der 12-Hydroxystearinsäure. Die Retentionszeit des letzteren war nur wenig kürzer als die des ersteren. Das Gemisch beider konnte praktisch nicht getrennt werden. Nach Sättigung der Säule erschien der Rizinolester in der Registrierkurve als sehr symmetrischer Peak. Die quantitative Auswertung ergab, dass die vom

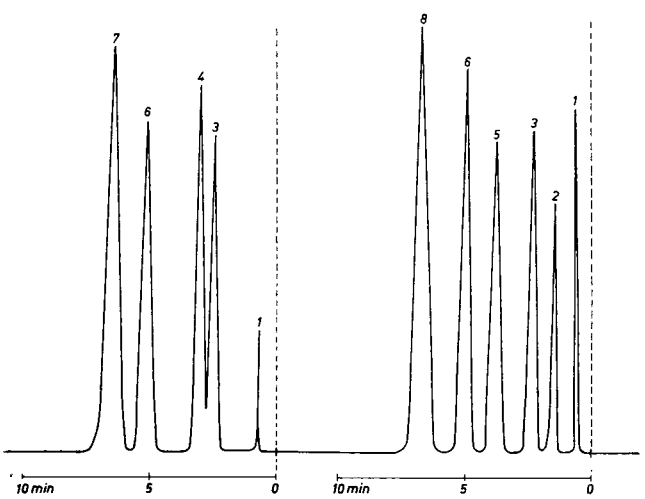

Fig. 3. Fettsäuremethylester. Temperatur 200°; Säulenlänge 2 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 3.3 l/Std. 1 = Diäthyläther; 2 = Capron-; 3 = Capryl-; 4 = Octen-4,5-; 5 = Caprin-; 6 = Undecan-; 7 = Undecen-10,11-; 8 = Laurin-säure.

TABELLE II

METHYLESTER *n*-FETTSÄURE

Fettsäure-methylester	*Einwaage (Gew. %)*	*Gefunden (Gew. %)*		*Einwaage (Gew. %)*	*Gefunden (Gew. %)*		*Einwaage (Gew. %)*	*Gefunden (Gew. %)*	
C_6				6.2	5.8	6.5			
C_7				10.1	10.0	10.4	5.2	5.7	4.9
C_8	12.8	13.5	13.8	9.3	8.6	8.4	10.1	9.3	9.9
$C_8^=$	17.3	16.4	16.6	15.7	16.3	16.1	9.3	10.2	10.0
C_9				4.1	3.9	4.3			
C_{10}				16.2	16.6	16.9	6.5	7.1	6.9
C_{11}	26.5	27.5	27.0	12.9	13.7	13.9	14.3	14.8	13.3
$C_{11}^=$	43.4	42.6	42.6	25.5	25.1	23.5	6.2	5.1	7.1
C_{12}							13.8	13.8	13.6
C_{13}							20.4	20.6	21.0
C_{14}							14.2	13.4	13.3

Schreiber umschriebenen Peakflächen direkt proportional den dosierten Mengen waren. Fig. 4 zeigt den Kurvenzug eines Testgemisches der Methylester der geradzahligen *n*-Fettsäuren von C_{14}–C_{22}, $C_{18}^=$, $C_{18}^{==}$, $C_{18}^{===}$ und der Rizinolsäure. Die Auswertung von 2 Gemischen ist in den vorderen beiden Spalten der Tabelle III enthalten. Die quantitativen Werte lagen auch bei diesen Gemischen innerhalb der üblichen Fehlergrenzen der GC-Analyse. Fig. 5 zeigt das Chromatogramm eines mit Methanol unter Zusatz von wenig Alkali umgeesterten Chin. Rizinusöles. Wir esterten nach dieser Methode mehrere Rizinusöle verschiedener Herkunft um und analysierten sie direkt gaschromatographisch. In Tabelle III sind die %-Gehalte der im Chin. Rizinusöl enthaltenen Fettsäuren angeführt. Die von uns erhaltenen Ergebnisse stimmten im wesentlichen mit denen von BINDER, APPLEWHITE, KOHLER UND GOLDBLATT[7] überein (über diese Arbeiten von uns wird an einer anderen Stelle im

TABELLE III

n-FETTSÄUREMETHYLESTER UND RIZINOLSÄUREMETHYLESTER

Fettsäure-methylester	*Ein-waage (Gew. %)*	*Gefunden (Gew. %)*		*Ein-waage (Gew. %)*	*Gefunden (Gew. %)*		*Ein-waage (Gew. %)*	*Gefunden (Gew. %)*		*Rizinusöl mit Methanol und Na umgeestert (Gew. %)*
C_{12}	2.0	2.3	1.9	6.2	5.9	6.5	3.6	3.2	3.8	
C_{14}	1.6	1.7	1.4	5.3	5.8	5.7	7.1	7.6	7.2	
C_{16}	3.2	3.6	3.0	6.1	6.6	6.0	5.4	5.0	5.8	1.1
$C_{16}^=$	0.9	0.7	1.0	1.9	1.5	2.1				0.2
C_{18}	1.7	1.6	2.0	8.2	7.8	8.3	3.6	2.9	3.2	1.2
$C_{18}^=$	3.2	3.8	3.6	10.0	9.3	9.7	4.5	5.6	4.9	2.3
$C_{18}^{==}$	4.8	5.2	4.5	16.7	17.4	17.2	2.9	2.7	3.3	4.7
$C_{18}^{===}$	1.3	0.9	0.9	6.3	5.7	5.6	3.8	4.1	3.4	0.9
C_{20}	1.8	1.5	1.7	6.9	6.8	7.3	7.2	6.7	6.9	1.0
C_{22}	2.1	2.7	2.7	8.5	9.2	9.6	3.1	3.9	3.8	
C_{18}=OH	77.4	75.0	77.3	23.9	24.0	22.0	58.8	58.3	57.7	88.6

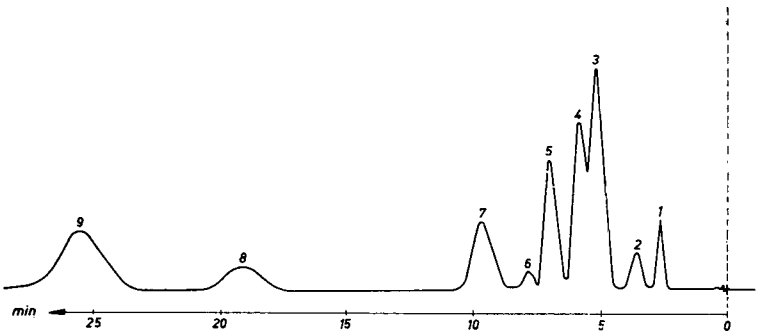

Fig. 4. Fettsäuremethylester. Temperatur 200°; Säulenlänge 2 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 11.6 l/Std. 1 = Myristin-; 2 = Palmitin-; 3 = Stearin-; 4 = Öl-; 5 = Linol-; 6 = Linolen-; 7 = Arachin-; 8 = Behen-; 9 = Rizinolsäuremethylester.

Zusammenhang mit der *Alkali*spaltung der Rizinolsäure berichtet werden). Zur quantitativen Auswertung letzterer Chromatogramme, die mittels Leitfähigkeitsmesszellendetektion gewonnen wurden, mussten unbedingt Testchromatogramme mit eingewogenen Gemischen angefertigt werden, da sich die von gleichen Gewichtsmengen Fettsäure- und Rizinolsäuremethylester als Nichthomologe erhaltenen Bergflächen wesentlich unterschieden.

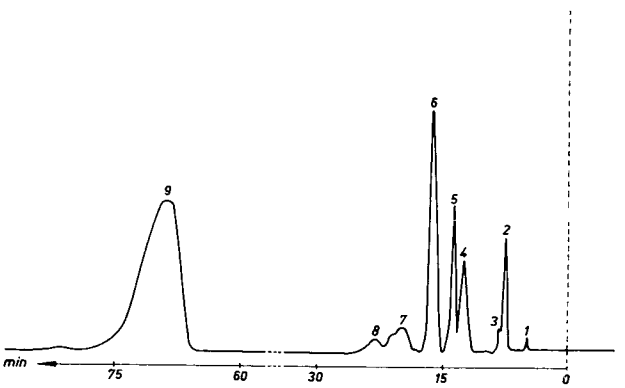

Fig. 5. Mit Methanol umgeestertes Rizinusöl. Temperatur 212°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 8.8 l/Std. 1–8 E = 1:1; 9 E = 1:5 (E = Empfindlichkeit). 1 = Myristinsäure; 2 = Palmitinsäure; 3 = Palmitoleinsäure; 4 = Stearinsäure; 5 = Ölsäure; 6 = Linolsäure; 7 = Linolensäure; 8 = Arachinsäure; 9 = Rizinolsäure; alle als Methylester.

4. Dicarbonsäuren

Die Dicarbonsäuren wurden zur Trennung an der Ketosäurepolyestersäule ebenfalls in die Dimethylester übergeführt. Fig. 6 zeigt ein Chromatogramm der veresterten Homologen der Dicarbonsäuren im Kettenlängenbereich von C_4 bis C_{12} und die Tabelle IV zeigt die quantitative Auswertung zweier eingewogener Testgemische C_4–C_{10} und C_{12} sowie der geradzahligen Dicarbonsäuren C_4–C_{12}. Die Trennung war vollständig. Die einzelnen Komponenten wurden in symmetrischen Kurven-

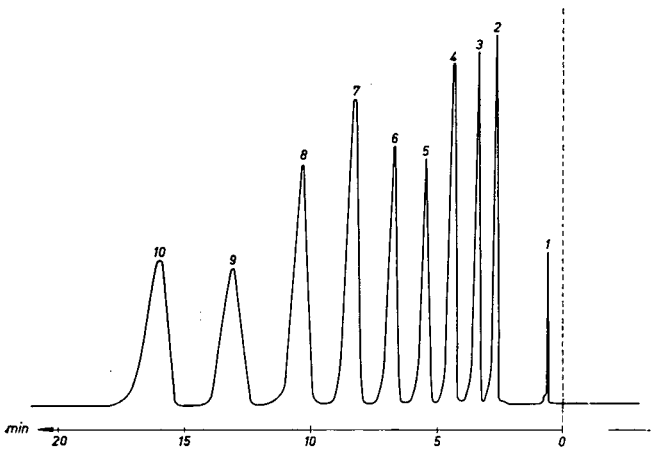

Fig. 6. Methylester der gesättigten Dicarbonsäuren. Temperatur 216°; Säulenlänge 4 m, ø 6 mm; Saulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 9.7 l/Std. 1 = Diäthyläther; 2 = C_4; 3 = C_5; 4 = C_6; 5 = C_7; 6 = C_8; 7 = C_9; 8 = C_{10}; 9 = C_{11}; 10 = C_{12}.

TABELLE IV

n-DICARBONSÄURE-DIMETHYLESTER

Disäure-dimethylester	*Einwaage (Gew. %)*	*Gefunden Höhe × $^1/_2$ Peakbreite*		*Gefunden mit Planimeter*		*Einwaage (Gew. %)*	*Gefunden Höhe × $^1/_2$ Peakbreite*		*Gefunden mit Planimeter*
C_4	7.6	7.4	7.1	7.3	8.0	8.1	8.3	8.0	7.8
C_5	9.4	9.8	9.6	9.7	9.3				
C_6	12.1	12.6	12.5	12.4	11.9	17.8	16.9	17.1	17.3
C_7	8.7	9.2	8.9	8.8	9.1				
C_8	11.1	10.7	10.6	10.9	11.0	25.0	24.8	25.4	25.6
C_9	18.3	17.8	18.0	17.8	17.9				
C_{10}	17.1	16.4	17.3	17.5	18.0	25.9	25.9	25.6	25.5
C_{12}	15.7	15.1	16.0	15.6	14.8	23.2	24.1	23.9	23.8

zügen eluiert und liessen sich einfach nach der Methode Peakhöhe × mittlere Breite auswerten.

5. Acetate der Fettalkohole

Die Acetate der längerkettigen Alkohole verhielten sich analog wie die Fettsäuremethylester. Am schlechtesten war auf unserem γ-Ketosäurepolyester die Trennung Stearylacetat von Oleylacetat. Die kürzeren, kettengleichen, gesättigten und ungesättigten Alkoholacetate wurden hingegen recht gut aufgetrennt. Fig. 7 ist das aufgenommene Chromatogramm eines Modellgemisches der Acetate der gesättigten Fettalkohole mit 10–20 Kohlenstoffatomen. Im vorderen Teil der Tabelle V ist die quantitative Auswertung enthalten. Nach beiden Auswertungsmethoden wurde eine befriedigende Übereinstimmung mit den Werten der Testgemische gefunden. Fig. 8 zeigt das Gemisch der geradzahligen Alkoholacetate C_{10}–C_{20} mit den

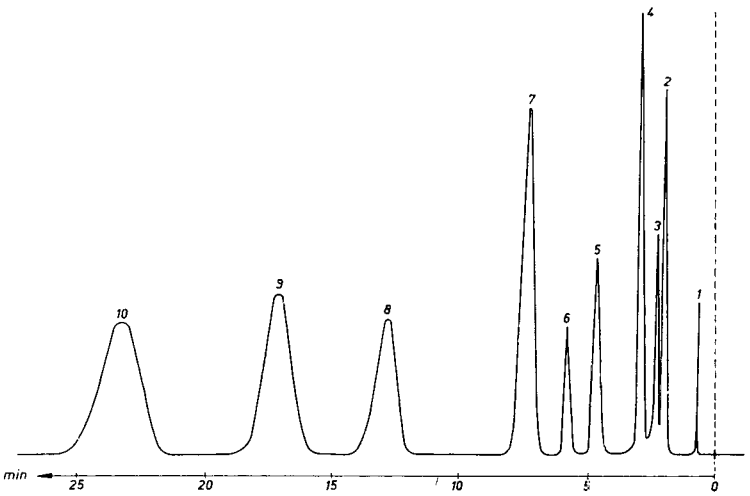

Fig. 7. Acetate der *n*-Alkohole. Temperatur 216°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 9.8 l/Std. 1 = Diäthyläther; 2 = Decanol; 3 = Undecanol; 4 = Dodecanol; 5 = Tetradecanol; 6 = Pentadecanol; 7 = Hexadecanol; 8 = Octadecanol; 9 = Nonadecanol; 10 = Eikosanol.

Acetaten von Palmitoleylalkohol, Oleylalkohol, Linolylalkohol und Linolenylalkohol, und im hinteren Teil der Tabelle V ist die quantitative Auswertung eines Modellgemisches der gleichen Acetate enthalten. Obwohl die gesättigten und ungesättigten kettengleichen Verbindungen nicht völlig aufgetrennt wurden, war die quantitative Auswertung noch recht befriedigend.

TABELLE V

ACETATE DER *n*-ALKOHOLE

Acetat des Alkohols	*Einwaage (Gew. %)*	*Gefunden Höhe × 1/2 Breite*		*Gefunden mit Planimeter*		*Einwaage (Gew. %)*	*Gefunden Höhe × 1/2 Breite*		*Gefunden mit Planimeter*	
C_{10}	3.1	3.3	2.9	3.2	3.0	3.0	3.4	3.1	3.5	3.2
C_{11}	2.0	2.4	2.6	2.5	2.5					
C_{12}	7.1	6.9	6.6	6.8	6.8	3.2	2.9	3.4	3.0	3.5
C_{14}	5.3	5.7	5.1	5.7	5.3	5.8	5.5	5.8	5.6	5.7
C_{15}	3.9	4.2	4.5	4.1	4.6	?	0.8	0.7	0.9	0.7
C_{16}	18.9	18.1	19.3	18.0	19.1	12.5	12.0	12.1	11.9	12.1
$C_{16}^{=}$						5.7	5.2	5.0	5.1	5.2
C_{18}	15.6	16.2	15.8	16.0	15.4	9.9	8.8	9.3	9.2	9.5
$C_{18}^{=}$						13.7	14.6	12.9	14.8	13.1
$C_{18}^{==}$						9.5	9.9	10.4	10.0	10.6
$C_{18}^{===}$						10.2	10.9	10.1	10.8	10.6
C_{19}	20.2	19.1	20.5	20.7	20.3					
C_{20}	23.9	24.1	22.7	23.1	23.0	26.5	26.0	27.2	25.2	25.8

6. Freie Fettalkohole

Die Trennung der niederen Alkohole war schon oft Gegenstand gaschromatographischer Untersuchungen. Es soll an dieser Stelle nicht weiter darauf eingegangen

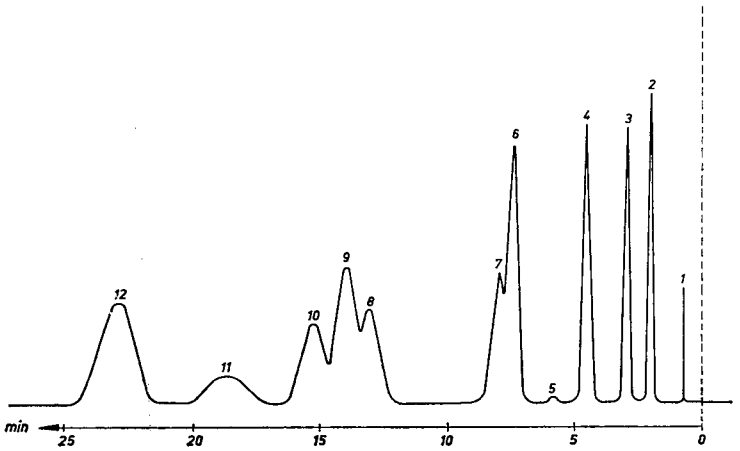

Fig. 8. Acetate der *n*-Alkohole. Temperatur 216°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 9.8 l/Std. 1 = Diäthyläther; 2 = Decanol; 3 = Laurylalkohol; 4 = Myristinalkohol; (5 = Pentadecanol); 6 = Palmitylalkohol; 7 = Palmitoleyalkohol; 8 = Stearylalkohol; 9 = Oleylalkohol; 10 = Linolylalkohol; 11 = Linolenylalkohol; 12 = Arachylalkohol.

und lediglich erwähnt werden, dass sich mit unserer Verteilungsphase die mittleren kettengleichen gesättigten und ungesättigten Alkohole recht gut voneinander trennen liessen. Wie bei allen anderen polaren Phasen traten die gesättigten zuerst aus der Säule aus. An einer 4 m Säule erhielten wir bei 100° eine vollkommene Trennung von Hexan-1-ol und 5,6-Hexen-1-ol und ebenfalls der beiden analogen Alkohole der Kettenlänge C_8 bei 130°. Die Peaks waren völlig symmetrisch und zeigten im Gegensatz zu den Elutionskurven in Chromatogrammen an den stärksten polaren unsubstituierten Polyestern praktisch kein "tailing".

Mit unserer Liquid-Phase gelang jedoch keine restlose Auftrennung der 4 Stellungsisomeren Octanole. Die Trennung der 3 Alkohole Octan-1-ol, Octan-2-ol und Octan-3-ol voneinander war vollständig, bei einer Säulenlänge von 4 m und einer Temperatur von 120°. Trotz Variierung von Temperatur, Säulenlänge und Gasflussgeschwindigkeit verliefen die Trennungen von Octan-3-ol und Octan-4-ol nicht restlos.

Die Retentionszeit lag am kürzesten bei 4-Stellung der Hydroxylgruppe. Der grösste Retentionsunterschied war zwischen Octan-1-ol und Octan-2-ol zu beobachten und führte so zur vollständigen Isolierung. Unter vergleichbaren Bedingungen gelang die Trennung dieser drei Alkohole an allen anderen von uns eingesetzten Polyesterphasen nicht ausreichend. Die Retentionszeiten waren wesentlich länger, während sich die Retentionszeitdifferenz zwischen Octan-1-ol und Octan-2-ol auf die knappe Hälfte verkürzte. Hinzu kam noch der schon erwähnte starke "tailing"-Effekt der Elutionskurvenzüge. Die günstige Trennleistung der Äthylenglykol-γ-ketopimalet-polyesterphase für diese Alkohole ist nach unserer Ansicht auf die starke Dipolwechselwirkung der Hydroxylgruppe des primären Alkohols mit der der freien Ketogruppe des Polyesters zurückzuführen.

Durch die Methylgruppe des Octan-2-ol erfolgt eine wesentliche Abschwächung des Dipol–Dipol-Effektes durch Abschirmung. Diese verstärkt sich dann weit weniger,

wenn die Hydroxylgruppe weiter ins Innere des Moleküls rückt und der abschirmende aliphatische Rest länger wird. Hierdurch werden die Retentionswerte der Octan-3,4-ole immer ähnlicher, es erfolgt keine restlose Auftrennung mehr unter unseren Bedingungen.

Dieser Abschirmungseffekt wurde auch von SCHOMBURG[8] beschrieben bei der gaschromatographischen Analyse von Fettsäuremethylestern, die alle die gleiche Kettenlänge besassen und in denen die Estergruppen an verschiedenen Stellen sich befanden. Durch die grössere Trennleistung der vom Verfasser verwandten Kapillarsäulen erfolgte eine wesentliche bessere Auftrennung der Gemische. Der grösste Retentionszeitunterschied war nach dem genannten Autor auch bei diesen Verbindungen zwischen der 1- und 2-Stellung der Estergruppe zu beobachten.

Die Liquid-Phase bewährte sich gut bei der Trennung länger- und langkettiger Fettalkohole. Letztere wurden von uns gewonnen durch Reduktion mit $Li(AlH_4)$ von G.C.-reinen Fettsäureestern. Fig. 9 zeigt die Elutionskurven eines Modellgemisches der primären *n*-Alkohole mit 11, 12, 14, 15, 16, 18, 19 und 20 C-Atomen und die Fig. 10 die eines Modellgemisches, wie wir es zur Auswertung der Chromatogramme von Ocenolen verwenden. Fig. 11 wurde erhalten bei der gaschromatographischen Analyse eines Ocenols, dem zur Erhöhung der Doppelbindungszahl Linolylalkohol zugesetzt worden war. Diesem Produkt wurden von uns für die quantitative Auswertung eingewogene Mengen von Pentadecanol und Heptadecanol zugesetzt.

In Tabelle VI sind die quantitativen Ergebnisse von 2 komplexen Alkoholgemischen enthalten. Die Auswertung erfolgte immer bei unveresterten Alkoholen gegen Eichchromatogramme, da wir Wärmeleitfähigkeitsmesszellen als Detektoren verwandten.

Durch verschiedene Versuchsreihen wurde von uns festgestellt, dass die Bedingung von der Proportionalität der Menge zur Bergfläche gut erfüllt ist, wie aus der Tabelle VII zu ersehen ist.

An der Polyesterphase konnten von uns sehr gut die 1,2-Dihydroxypropyl-*n*-alkyläther auf ihre Reinheit geprüft werden. Fig. 12 enthält das Gaschromatogramm

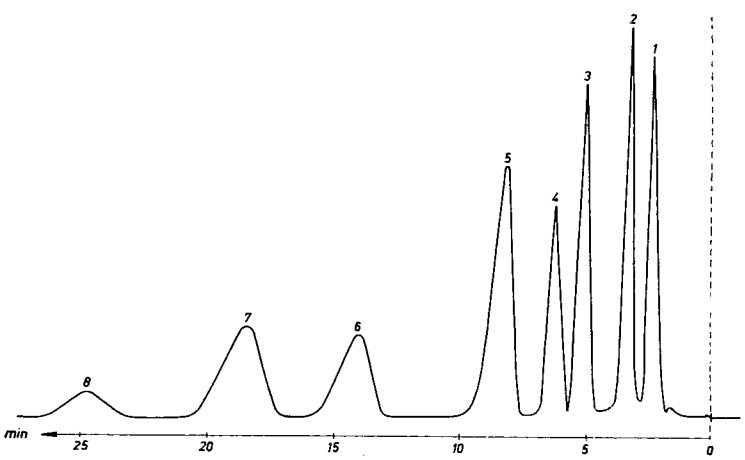

Fig. 9. Gesättigte *n*-Alkohole. Temperatur 211°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 10.8 l/Std. 1 = C_{11}; 2 = C_{12}; 3 = C_{14}; 4 = C_{15}; 5 = C_{16}; 6 = C_{18}; 7 = C_{19}; 8 = C_{20}.

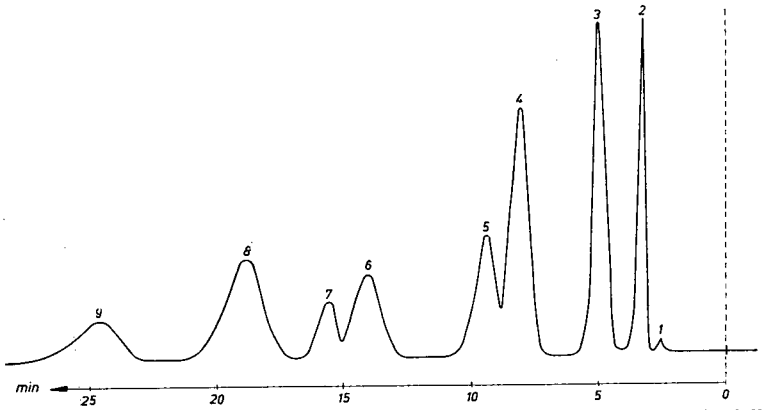

Fig. 10. *n*-Alkohole. Temperatur 211°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 10.8 l/Std. 1 = Undecanol; 2 = Dodecanol; 3 = Tetradecanol; 4 = Hexadecanol; 5 = 8,9-Hexadecen-1-ol; 6 = Stearylalkohol; 7 = Oleylalkohol; 8 = Linolylalkohol; 9 = Eikosanol.

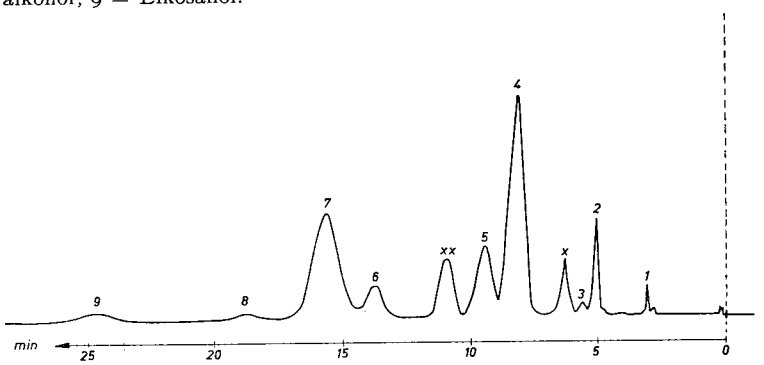

Fig. 11. Gesamtocenol. Temperatur 211°; Säulenlänge 4 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 10.8 l/Std. 1 = Dodecanol; 2 = Tetradecanol; 3 = Tetradecenol; 4 = Hexadecanol; 5 = Hexadecenol; 6 = Stearylalkohol; 7 = Oleylalkohol; 8 = Linolylalkohol; 9 = Eikosanol; x = Testsubstanz Pentadecanol; xx = Testsubstanz Heptadecanol.

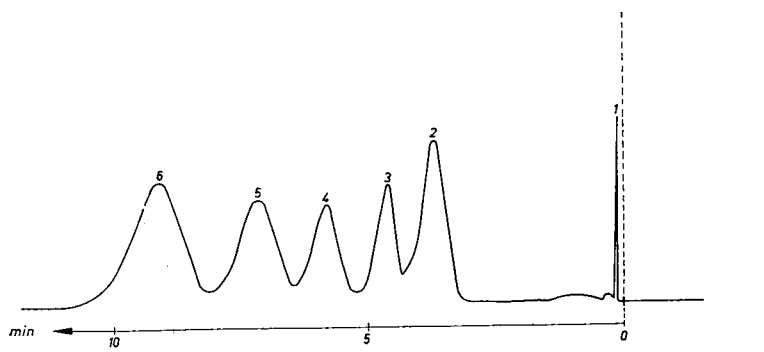

Fig. 12. 1,2-Dihydroxypropyl-*n*-Alkyläther mit *n*-Alkylrest. Temperatur 206°; Säulenlänge 1.58 m, ø 6 mm; Säulenfüllung 30% Polyester auf Sterchamol; Schleppgas H_2 8.1 l/Std. 1 = Diäthyläther; 2 = octyl; 3 = nonyl; 4 = decyl; 5 = undecyl; 6 = dodecyl als Alkylrest.

TABELLE VI

n-ALKOHOLE

Alkohol	*Einwaage (Gew. %)*	*Gefunden Höhe × 1/2 Breite*		*Gefunden mit Planimeter*		*Einwaage (Gew. %)*	*Gefunden Höhe × 1/2 Breite*		*Gefunden mit Planimeter*	
C_8	6.1	6.5	6.0	6.4	5.8	2.4	2.0	1.9	2.1	2.0
C_9	7.0	7.1	6.6	7.2	6.7					
C_{10}	8.2	8.0	8.7	8.1	8.0	4.7	5.2	5.0	5.1	5.1
C_{11}	5.9	5.9	5.8	5.8	5.9					
C_{12}	6.3	6.6	5.9	6.7	6.8	8.1	8.6	8.5	8.7	8.7
C_{14}	8.9	8.5	8.8	8.3	8.7	5.3	6.2	5.8	6.4	6.0
C_{15}	11.1	10.7	11.6	10.9	11.7					
C_{16}	10.9	11.6	11.4	11.4	11.1	18.1	17.4	17.8	17.3	17.8
$C_{16}^{=}$						10.0	9.6	9.0	9.5	9.1
C_{18}	13.6	13.1	13.0	13.3	13.4	8.2	8.7	9.1	8.5	9.0
$C_{18}^{=}$						16.1	15.1	15.8	15.4	16.0
$C_{18}^{==}$						5.5	6.5	6.2	6.6	6.0
C_{19}	9.7	10.1	9.6	10.0	9.9					
C_{20}	12.3	11.9	12.6	11.9	11.9	20.6	20.7	20.9	20.4	20.3

der Mischung dieser Substanzen mit den *n*-Alkylresten C_8–C_{12} und Fig. 13 das Diagramm der Logarithmen der Retentionsvolumina, aufgetragen gegen die Kohlenstoffanzahl. Die von JAMES UND MARTIN[9] gefundenen Beziehungen gelten auch für die homologe Reihe der Dihydroxypropyl-*n*-alkyläther. Mit dieser chromatographischen Analyse konnten wir den Beweis dafür erbringen, dass nach 3 verschiedenen präparativen Methoden gleiche Produkte entstanden waren und die Verätherung praktisch an der primären Hydroxylgruppe stattgefunden hatte, da die Äther mit den Alkylresten C_8 und C_{12} aus 1,2-Epoxypropyl-*n*-alkyläthern durch Spaltung des Epoxyringes entstanden waren. Die Epoxy-Äther waren nach dem U.R.-Spektrogramm frei von Hydroxylgruppen.

TABELLE VII

ERHALTENE PEAKFLÄCHE EINER ALKOHOLMENGE k × FAKTOR n

Alkohol	*Faktor n*					
	1	*2*	*4*	*7*	*10*	*20*
C_{11}	1	1.95	4.03	6.85	10.48	18.95
C_{12}	1	2.03	4.06	7.16	9.81	20.13
C_{14}	1	2.04	3.90	7.12	10.60	21.06
C_{15}	1	2.02	3.96	6.92	10.12	19.25
C_{16}	1	1.97	3.88	7.13	9.62	20.38
C_{18}	1	2.06	4.09	7.21	9.88	19.42
$C_{18}^{=}$	1	1.94	3.91	6.84	10.50	19.67
$C_{18}^{==}$	1	1.92	4.11	6.96	10.83	19.98
C_{20}	1	2.04	4.08	7.10	9.54	20.60
$C_{18}^{=OH}$	1	1.98	3.91	7.14	9.38	18.72

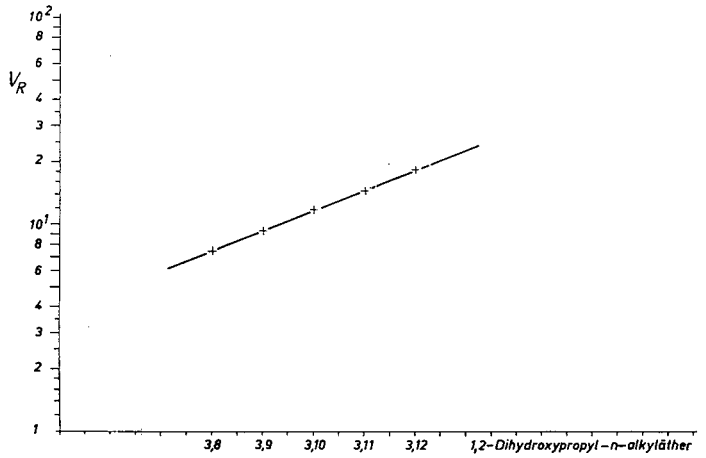

Fig. 13. Log (Retentionsvolumina) der 1,2-Dihydroxypropyl-*n*-Alkyläther gegen Anzahl der Kohlenstoffatome.

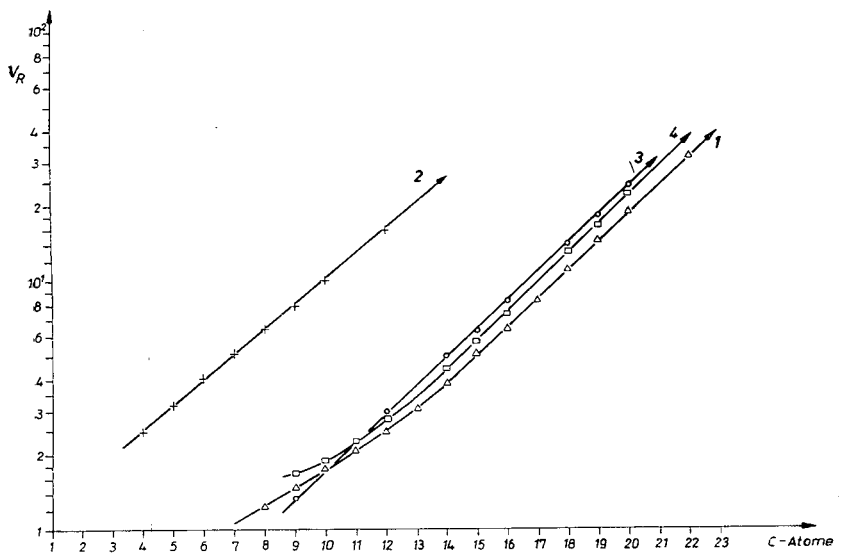

Fig. 14. Log (Retentionsvolumina) gegen Anzahl der Kohlenstoffatome. (C-Atome beziehen sich auf die unveresterten Verbindungen.) 1 = Fettsäuremethylester; 2 = Dicarbonsäuredimethylester; 3 = Fettalkohole; 4 = Fettalkoholacetate.

7. Retentionsvolumina

Die Tabelle VIII enthält die Retentionsvolumina von:

(a) Methylestern gesättigter und ungesättigter Fettsäuren bei 216°;
(b) Dimethylestern der Dicarbonsäuren bei 216°;
(c) Acetaten gesättigter und ungesättigter Fettalkohole bei 216°;
(d) gesättigten und ungesättigten Fettalkoholen bei 211°.

TABELLE VIII

RETENTIONSZEITEN

T = 216° *Methylester der n-Fettsäuren bez.*			*T* = 216° *Dimethylester der n-Dicarbonsäuren bez. auf* C_4		*T* = 216° *Acetate der n-Alkohole bez. auf* C_{14}		*T* = 211° *n-Alkohole bez. auf* C_{14}	
	auf C_{10}	*auf* C_{14}						
			C_4	1.00				
			C_5	1.27				
C_6	0.47	0.23	C_6	1.66				
C_7	0.56	0.27	C_7	2.08	C_7	0.26		
C_8	0.68	0.32	C_8	2.54	C_8	0.30	C_8	0.20
C_9	0.82	0.39	C_9	3.15	C_9	0.35	C_9	0.26
C_{10}	1.00	0.48	C_{10}	3.92	C_{10}	0.43	C_{10}	0.34
C_{11}	1.18	0.56	C_{11}	—	C_{11}	0.52	C_{11}	0.45
C_{12}	1.47	0.70	C_{12}	6.08	C_{12}	0.64	C_{12}	0.62
C_{13}	1.82	0.84						
C_{14}	2.10	1.00			C_{14}	1.00	C_{14}	1.00
C_{15}	2.88	1.37			C_{15}	1.26	C_{15}	1.25
C_{16}	3.71	1.77			C_{16}	1.64	C_{16}	1.62
C_{17}	4.82	2.30			C_{17}	—	C_{17}	—
C_{18}	6.41	3.05			C_{18}	2.82	C_{18}	2.80
C_{19}	8.59	4.09			C_{19}	3.80	C_{19}	3.64
C_{20}	11.47	5.46			C_{20}	5.14	C_{20}	4.80
$C_{18}{=}$	6.91	3.29			$C_{14}{=}$	1.12	$C_{16}{=}$	1.96
$C_{18}{=}{=}$	8.12	3.87			$C_{16}{=}$	1.89	$C_{18}{=}$	3.24
$C_{18}{=}{=}{=}$	10.90	5.19			$C_{18}{=}$	3.15	$C_{18}{=}{=}$	3.96
C_{18}=OH					$C_{18}{=}{=}$	3.45		
	37.50	17.86			$C_{18}{=}{=}{=}$	4.30		

In Fig. 14 sind die Logarithmen der Retentionsvolumina gegen die Kohlenstoffanzahl der Säurekomponente, der Dicarbonsäurekomponente und der Alkoholkomponente aufgetragen. Bei den stärker polaren Verbindungen (Alkoholen, Dicarbonsäuredimethylestern und Dihydroxypropyl-*n*-alkyläthern) liegen die Homologen praktisch auf Geraden. Die Kurven der schwächer polaren Methylester der Fettsäuren und Acetate der Fettalkohole zeigen in den niederen Gliedern eine Krümmung.

ZUSAMMENFASSUNG

Es wird die Herstellung und Verwendung einer Polyester-Liquid-Phase, die aus γ-Ketopimelinsäure und Äthylenglykol erhalten wurde, angegeben. Sie eignet sich besonders zur Trennung mittel- und langkettiger Fettalkohole. Die Retentionsvolumina sind wesentlich kürzer wie bei Verwendung unsubstituierter Polyesterphasen.

SUMMARY

Application and preparation of a polyester liquid phase, which was obtained from 4-oxopimelic acid and ethylene glycol, are described. It is especially suitable for the separation of middle- and long-chain fatty alcohols. The retention volumes are substantially smaller than they would be if unsubstituted polyester phases were used.

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ESSENTIAL OILS AND THEIR CONSTITUENTS

XXIV. STUDY OF SESQUITERPENE DEHYDROGENATION REACTIONS BY GAS-LIQUID CHROMATOGRAPHY*

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INTRODUCTION

Dehydrogenation of terpenoids into readily identifiable compounds has been frequently adopted for ascertaining their carbon frameworks[1]. Cyclic sesquiterpenoids yield often alkyl substituted naphthalenes and azulenes which can, in turn, be characterized *via* conversion into suitable crystalline derivatives, *e.g.* picrates and trinitrobenzene adducts[2].

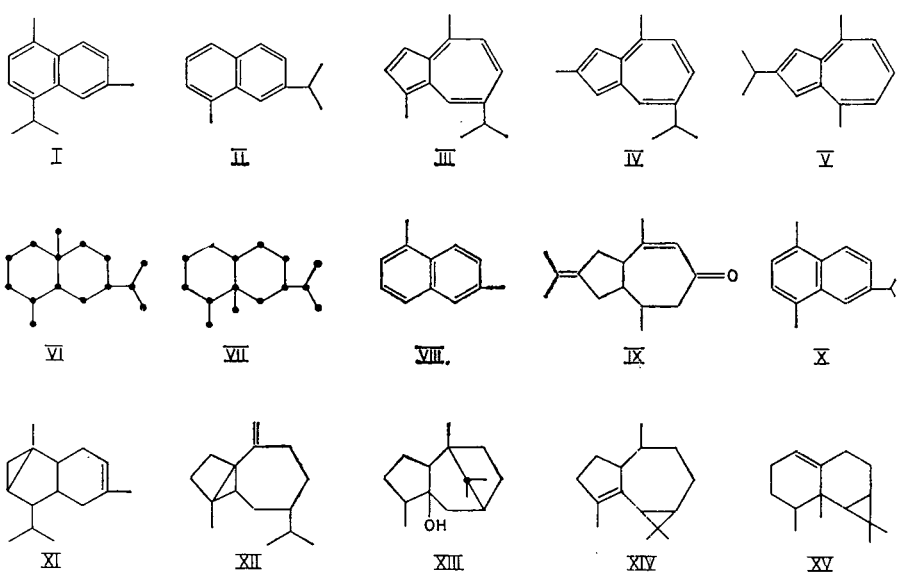

Fig. 1. Typical sesquiterpenoids and their dehydrogenation products.

Fig. 1 illustrates the structures of two substituted naphthalenes, cadalene (I) and eudalene (II), as well as three azulenes, S-guaiazulene (III), Se-guaiazulene (IV)

* Paper XXIII: *J. Pharm. Sci.*, 53 (1964) 1407.

and vetivazulene (V) frequently met with in such investigations. Cadalene, S-guaiazulene and vetivazulene are usually generated from parent compounds possessing the corresponding carbon skeletons. Eudalene, a C_{14} compound, may form from either VI or VII following elimination of the angular methyl group. Se-guaiazulene, on the other hand, is a product of rearrangement of S-guaiazulene which it usually accompanies when dehydrogenation of S-guaiazulene compounds is carried out over selenium at elevated temperatures. 1,6-Dimethylnaphthalene has been observed as a dehydrogenation product of isozingiberene[3].

Conventional dehydrogenations are rather drastic processes and often complex mixtures containing products of rearrangement as well as degradation are obtained. The resolution of such mixtures and identification of some of their constituents has been achieved through combination of extensive column chromatography and repeated recrystallization of their derivatives with picric acid and trinitrobenzene.

In the authors' laboratory, gas chromatography has been used successfully for some time to study the course of dehydrogenation reactions of sesquiterpenes found in essential oils. The technique proved to be a valuable analytical tool as complex mixtures were effectively resolved without recourse to tedious and time consuming operations of column chromatography, derivative formation and recrystallizations. It is the purpose of this paper to report some of the results obtained.

EXPERIMENTAL

(1) *Methods*

Dehydrogenation of sesquiterpene fractions. Selenium and sulphur were used as dehydrogenating agents. The experimental sample (250 mg) was weighed into a small bulb (3 ml). Selenium (500 mg) or sulphur (115 mg) was added and the assembly fused to an air condenser comprised of two concentric tubes. The mixture was refluxed for 24 h at 290 $\pm$ 10° (selenium dehydrogenation) or 200 $\pm$ 5° (sulphur dehydrogenation) while a slow stream of nitrogen gas was allowed to pass through the condenser. Azulenogenic sesquiterpenes were processed for only 4 h. Following reaction, the bulb was cut off, the product was dissolved in hexane, and chromatographed over 5 g of grade I basic alumina. The eluate, recovered following evaporation of the solvent, was examined by gas chromatography. Coloured effluents (azulenic hydrocarbons) were collected separately.

Gas chromatographic analysis. The products of dehydrogenation were examined by means of a column of Reoplex 400 (20 %) on acid-washed Chromosorb W. Experimental procedures have been described[4]. Reoplex 400 (10 %) and silicone gum SE-30 (10 %) columns were also used for some of the investigations. Collection of samples for spectral identification was carried out by either depositing effluents directly on a salt plate[5] or trapping in carbon tetrachloride.

(2) *Materials*

Products used as reference compounds were obtained in accordance with the procedures described and are listed in Table I. Acenaphthene and fluorene (commercial products) were used as internal standards.

TABLE I

PREPARATION OF REFERENCE COMPOUNDS

Compound	*Source*	*Method of preparation*
Cadalene	Cadinene fraction from oil of cade	Se-Dehydrogenation
Eudalene	Selinene fraction from oil of celery seed	Se-Dehydrogenation
S-Guaiazulene	Gurjunene fraction from oil of gurjun balsam	S-Dehydrogenation
Se-Guaiazulene	Gurjunene fraction from oil of gurjun balsam	Se-Dehydrogenation
Vetivazulene	Sesquiterpene fraction from oil of vetiver	Se-Dehydrogenation

RESULTS AND DISCUSSION

The gas chromatographic behaviour of naphthalenes and azulenes—see Fig. 1 (I to V and VIII)—was investigated using a polar (Reoplex 400; 20 %) and a non-polar (Silicone gum SE-30; 10 %) column. The six compounds were well separated by either substrate. Retention times were higher on the Reoplex column and a temperature of 220° had to be used to elute the compounds within 30 min. The SE-30 column could be conveniently operated at 200°.

For the separation of naphthalenes, lower column temperatures—200° for Reoplex and 185° for SE-30—were found to improve resolution, and for the separation of the high boiling azulenes a 10 % Reoplex column was found to be preferable.

Fig. 2-A illustrates the separation of a six-component reference mixture by a Reoplex column operated at 220°, and Table II shows the relative retention times of these compounds using both the Reoplex and SE-30 substrates. Fluorene and acenaphthene served as reference standards. Both these compounds displayed gas chromatographic peaks which did not overlap with any of the reaction products.

As may be seen from Table II, Reoplex 400 proved the more efficient substrate. It retained aromatics more strongly than the corresponding alicyclic and aliphatic

TABLE II

RELATIVE RETENTION TIMES OF SESQUITERPENE DEHYDROGENATION PRODUCTS

Compound	*B.P.* (°C)*	*Retention times* (*Helium flow rate: 75 ml/min*)			
		SE-30		*Reoplex*	
		*200°***	*185°****	*220°***	*200°****
1,6-Dimethyl-naphthalene	262–263	0.61	0.81	0.42	0.67
Eudalene	281	0.90	1.27	0.50	0.82
Acenaphthene	279	0.73	1.00	0.63	1.00
Cadalene	292	1.30	1.93	0.70	1.20
Fluorene	295	1.00	1.40	1.00	1.71
S-Guaiazulene	176/17 mm	1.89	—	1.17	—
Se-Guaiazulene	170–171/13 mm	1.95	—	1.26	—
Vetivazulene	—	2.08	—	1.37	—

* Literature values.
** Relative to fluorene.
*** Relative to acenaphthene.

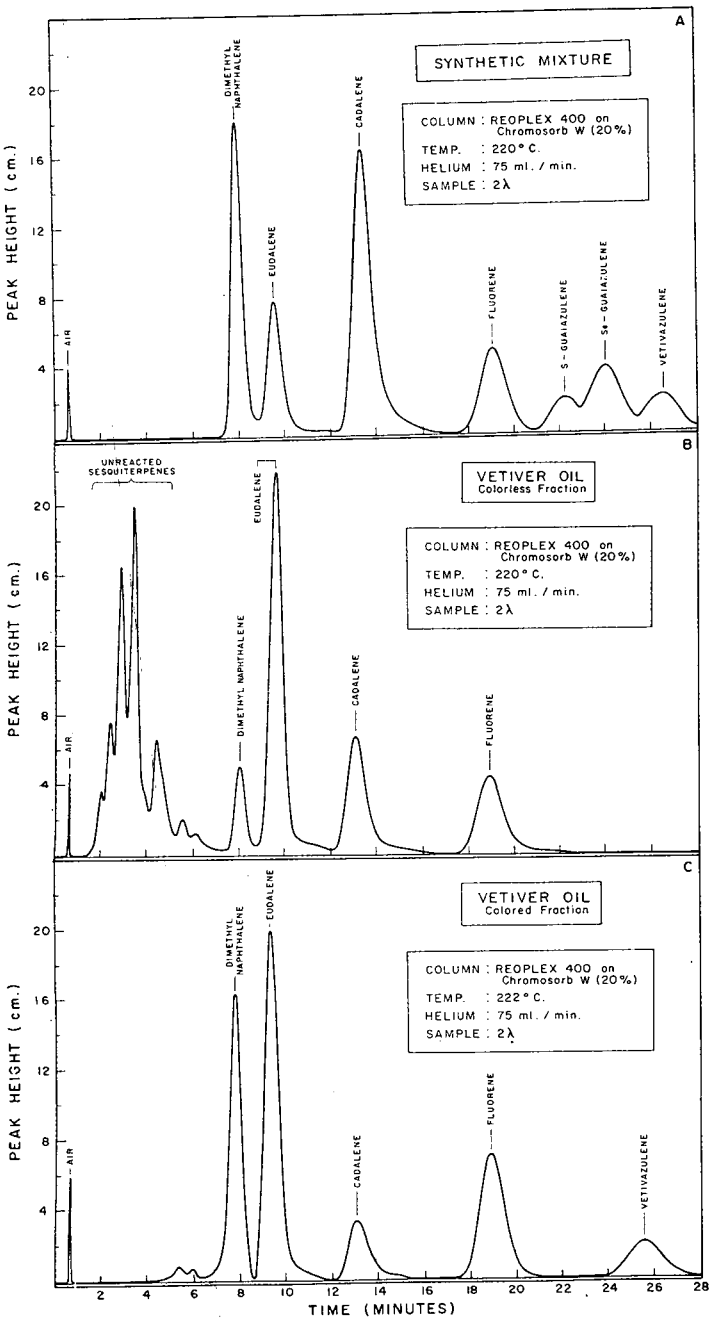

Fig. 2. Gas chromatography of sesquiterpene dehydrogenation products. Reference standard: fluorene.

compounds. The non-polar SE-30 column lacked such selectivity, its resolving properties being determined primarily by the vapour pressures of the different constituents. The Reoplex column was, therefore, found to be suited for the separation of unchanged sesquiterpenes from their aromatic dehydrogenation products. It also separated effectively lower boiling naphthalenes, formed by cracking of the larger molecules during the drastic process of dehydrogenation, from both the unreacted sesquiterpenes and their primary dehydrogenation products.

The stability of the compounds isolated under the experimental conditions described was confirmed by systematic comparison of the infrared spectra of effluents with those obtained following re-chromatography and similar analysis of the condensates collected.

The following examples serve to illustrate the scope and application of the technique.

Resolution of complex mixtures

The sesquiterpene hydrocarbon fraction of vetiver oils is known to contain compounds of various carbon frameworks including cadalenic, eudalenic and azulenic substances[6]. On dehydrogenation it yields a complex mixture of aromatic and azulenic constituents. Fig. 2-B and 2-C show how readily such a complex mixture can be analysed by gas–liquid chromatography since unreacted sesquiterpenes elute much faster than their aromatic as well as azulenic degradation products.

Dealkylations associated with dehydrogenation reactions

The earliest known example of dealkylation occurring during aromatization of sesquiterpenes is the formation of eudalene from selinene and related sesquiterpenes following elimination of the angular methyl group[7]. Dealkylation at other positions has also been observed[8]. SEIDEL *et al.* reported formation of a small quantity of 1,6-dimethylnaphthalene during dehydrogenation of isozingiberene[3]. We obtained good yields (66 %) of a low-boiling compound when preparing cadalene from cadinene fractions of cade oil in accordance with the procedure described. The infrared spectrum of this compound displayed absorptions characteristic of substituted naphthalenes. Rigorous comparison of spectral and retention time data with those of authentic samples of naphthalenes thought to be present proved the substance to be 1,6-dimethylnaphthalene. The compound was subsequently found to accompany cadalene in dehydrogenation products derived from the sesquiterpene fractions of many essential oils. This observation is in accord with the view of SEIDEL *et al.* that 1,6-dimethylnaphthalene is formed by cracking of the cadalene molecule.

Rearrangement of dehydrogenation products

Under the drastic reaction conditions of conventional dehydrogenations, the primary products formed may also rearrange to compounds possessing entirely different carbon skeletons. Examples are the formation of Se-guaiazulene from S-guaiazulene owing to migration of a methyl group, dehydrogenation of vetivones (IX) to vetivazulene, eudalene and vetivalene (X)[9], formation of naphthalenic and azulenic compounds from tricyclic substances, *e.g.* cadalene from copaene (XI)[10] and S-guaiazulene from aromadendrene (XII)[11] and patchoulol (XIII)[12].

A fraction of gurjun balsam oil containing α-gurjunene (XIV) and β-gurjunene

(XV) was dehydrogenated using selenium and sulphur. Gas chromatographic analysis of the reaction products showed that—as expected—selenium dehydrogenation generated both S-guaiazulene and Se-guaiazulene whereas sulphur dehydrogenation produced only S-guaiazulene. In addition to these azulenes, the presence of small quantities of cadalene, eudalene and 1,6-dimethylnaphthalene was also detected. It would appear, therefore, that eudalene originates from both α- and β-gurjunene and that cadalene, formed by rearrangement of α-gurjunene, also yields some 1,6-dimethylnaphthalene owing to cracking of its carbon skeleton.

ACKNOWLEDGEMENTS

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SUMMARY

The application of gas–liquid chromatography to the analysis of complex mixtures formed by dehydrogenation of sesquiterpenes is described. The usefulness of the technique in studying degradation and rearrangements accompanying such reactions is also illustrated.

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CHROMATOGRAPHIC STUDIES ON ORGANO-TIN COMPOUNDS

PART II. THE REACTIONS OF TRIMETHYLSTANNANE

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Although considerable work has been published on organo-stannanes, it has mainly concerned the aromatic stannanes, *e.g.* triphenylstannane. A review of organo-tin chemistry was compiled by INGHAM, ROSENBERG AND GILMAN[1], while further reviews by LUITJEN, VAN DER KERK AND NOLTES[2,3] and NEUMANN[4] have also been published.

This paper describes the preparation of trimethylstannane, and its reactions with organic halides, sulphides, amines, alcohols, olefins and acetylenes. The products of each reaction were identified and investigated by infra-red spectroscopy, gas–liquid chromatography, distillation and refractive index techniques:

PREPARATION OF TRIMETHYLSTANNANE

Trimethylstannane was prepared by the normal three-stage synthesis:

(a) Reaction of stannic chloride with an excess of methyl magnesium iodide in diethyl ether, under the usual Grignard conditions. After refluxing, the resultant mixture was baked until all the volatile material had been collected. (The usual procedure of the addition of water, to destroy the excess Grignard reagent was not followed, since it was found to lower the yield of tetramethylstannane, and to produce a large quantity of unidentified higher boiling-point compounds.) The tetramethyl-stannane was fractionated from the mixture at 78°. The purity of the product was checked by gas chromatography.

(b) Trimethylbromostannane was then prepared from tetramethylstannane by addition of the stoichiometric concentration of bromine, and refluxing in diethyl ether. The bromo-compound was purified by distillation.

(c) Trimethylstannane was prepared by the method due to FINHOLT *et al.*[5,6] and VAN DER KERK *et al.*[7]. Trimethylbromostannane was added slowly to an excess of lithium aluminium hydride in di-*n*-butyl ether (in an atmosphere of nitrogen) at room temperature. After gentle refluxing, the stannane was distilled at 59° from the mixture, and stored under nitrogen.

REACTIONS OF TRIMETHYLSTANNANE

The reactions of trimethylstannane were carried out using stoichiometric quantities of reagents, under an atmosphere of nitrogen. Usually, the mixtures were

gently refluxed to produce reaction, but in some cases, higher temperatures and pressures were employed by using sealed-glass tubes.

Reaction with organic halides

Trimethylstannane reacted exothermally with aliphatic bromides and aliphatic or aryl iodides, to produce the corresponding hydrocarbon and the trimethylhalogenostannane:

$$RX + Me_3SnH \longrightarrow RH + Me_3SnX \qquad (1)$$

R = alkyl or aryl group; X = halogen

Chloro-compounds and aryl bromides, however, reacted only after prolonged refluxing, giving small yields of products. These experiments confirmed the observations of ROTHMAN AND BECKER[8,9] that the reactivity of the tin–hydrogen bond towards alkyl halides increases in the order RCl > RBr > RI. The reaction mechanism of such stannane reactions was first thought to be ionic[10,11], the tin-hydrogen bond being polarised in the direction $Sn^{\delta+} \longrightarrow H^{\delta-}$; and this has been used to explain the greater reactivity of alkyl iodides than alkyl chlorides. However, recent observations reported by KUIVILA, MENAPACE AND WARNER[12] indicate that the reactions of trimethylstannanes occur through a radical mechanism, the rate-determining step being the formation of radicals from the organic halides, and further evidence for this theory is the catalytic effect of azoisobutyronitrile on such reaction systems[13]. The addition of trimethylstannane to olefins also indicates a free radical mechanism.

Reaction with alcohols, sulphides or amines

On refluxing alcohols or mercaptans with trimethylstannane, small amounts of the corresponding addition compounds are produced. Such compounds rapidly decompose in air, the reactions may be represented as follows:

$$Me_3SnH + Et\binom{O}{S}H \longrightarrow Me_3Sn\binom{O}{S}Et + H_2 \qquad (2)$$

However, in the case of disulphides, primary, secondary and tertiary amines, no reaction with trimethylstannane was observed, unlike the reaction of triphenylstannane with amines[11,14–17].

Reaction with olefins

Mixtures of trimethylstannane and olefins on refluxing give small yields of the corresponding addition compounds:

$$Me_3SnH + H_2C{=}CHR \longrightarrow Me_3SnCH_2CH_2R \qquad (3)$$

R = alkyl group

VAN DER KERK, NOLTES AND HENRY[18,19] suggested that the presence of a functional group or a conjugated double bond will facilitate the addition of unsaturated linkages to a tin–hydrogen bond. Such hydrostannation of olefins appears to proceed by a free radical mechanism[13,20]. It, therefore, seemed worthwhile to investi-

gate the behaviour of conjugated dienes in such a reaction. Piperylene (penta-1,3-diene) was readily available and it was decided to examine this reaction.

(*a*) *Piperylene.* Piperylene, together with other conjugated dienes[21], is readily hydrostannated at 60–85° by a radical mechanism. NEUMANN AND SOMMER[22] have shown that radical scavengers inhibit, and radical producers, *e.g.* azoisobutyronitrile, accelerate the reaction. BARNETSON, CLARK AND KWON[23] suggest that for the addition of dimethylstannane to conjugated dienes, their evidence indicates that it is the stannane which plays the major role and that formation of radicals from the olefin is not the rate-determining step. Further work since the publication of COOKE, NICKLESS AND POLLARD[24] now confirms this viewpoint.

Chromatographic analyses of the reaction mixture indicated the presence of four reaction products, and a typical chromatogram is shown in Fig. 1. The assignment of peaks is as follows: a = unchanged piperylene; b = tetramethylstannane; c, d, e, f = monoaddition products.

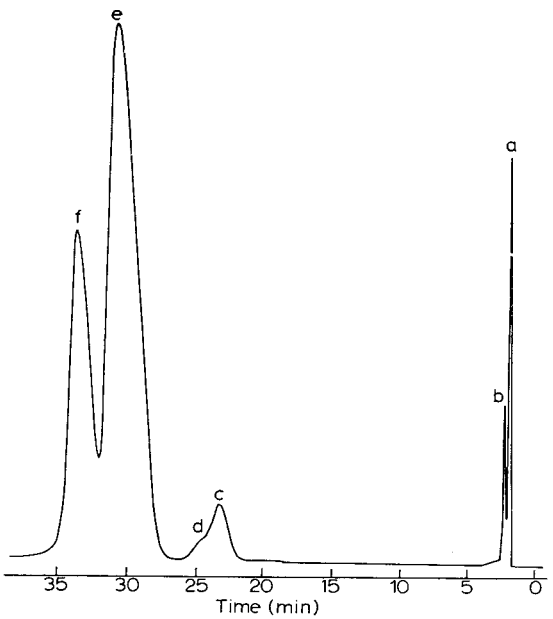
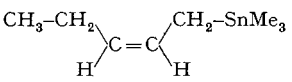


Fig. 1. Elution pattern for the trimethylstannane–piperylene reaction. a = piperylene; b = tetramethylstannane; c, d, e, f = monoaddition products.

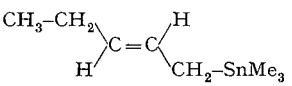
If these pentenyl compounds are formed, it is probably by simple 1,4- and 1,2-addition (compare ref. 24, where only 1, 2-addition was considered) to the conjugated system of piperylene. The configuration about the C=C bond is of great interest, since the following *cis* and *trans* isomers are possible, assuming addition of $-SnMe_3$ at the 1-carbon atom.

By considering the stereochemistry of the reaction, it was thought that addition of the $-SnMe_3$ group at carbon numbers 3 and 4 would be almost impossible because of steric hindrance. Also examination of the two doublet elution peaks suggested the presence of two sets of *cis* and *trans* isomers which would be expected if

1,4-addition

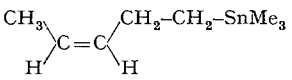


I (*cis*)

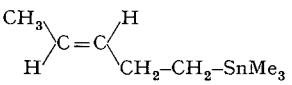


II (*trans*)

1,2-addition



III (*cis*)



IV (*trans*)

structures I–IV were involved. If, after the first addition of the trimethylstannyl group, only secondary carbon atoms in the conjugated system are left as radical centres, as in this case, then the yield of the 1,2-addition product rises from about 15 % to nearly 40 %.

$$Me_3Sn{-}CH_2{-}\dot{C}H{-}CH_2{=}CH{-}CH_3 \longrightarrow Me_3Sn{-}CH_2{-}CH{=}CH{-}\dot{C}H{-}CH_3$$

Of these major products, the product that gives peak e has structure II and f structure IV (a *trans* configuration is tentatively suggested because of the strong absorption of II at about 960 cm^{-1} and because of the relative sizes of the peaks). The minor peaks, c and d, may be the *cis* isomers I and III respectively, but this is thought unlikely because in general for the compound where *cis–trans* isomerism is possible, the *cis* isomer usually has a longer retention volume on the type of column used to separate these compounds than the *trans* isomer. This case is the direct converse of this situation. It is felt more likely that c and d are the inverse 4,1- and 2,1-addition products, respectively, but insufficient material is available at this time to allow any distinction between these postulates to be made.

(*b*) *1-Olefins*. In order to effect a higher degree of reaction, mixtures of trimethylstannane and 1-olefins were heated together at 100° under pressure in sealed glass tubes. The resultant products were analysed, and in each case the expected trimethylalkyl stannane, according to eqn. (3), was present, together with tetramethylstannane plus two compounds of intermediate boiling point which were unstable and decomposed very rapidly on exposure to air. The distribution of the products depended on the pressure, but it was found that the lower the pressure for a given pair of reactants, the larger the proportion of unstable compounds formed, and at relatively low pressures these compounds were present in 80 % yields.

The chromatograms of the reaction mixtures before and after the decomposition of the unstable compounds are given in Figs. 2 and 3. It proved impossible to identify the unstable products using infra-red trapping techniques. A clue to their identity can be obtained from the formation of tetramethylstannane, which can be produced by (a) thermal decomposition of trimethylhydroxystannane[25], (formed by the reaction between trimethylstannane and oxygen), or (b) the thermal decomposition of trimethylstannane[26]. It is extremely doubtful, however, whether these reactions are the cause of such a large proportion of tetramethylstannane being produced, since

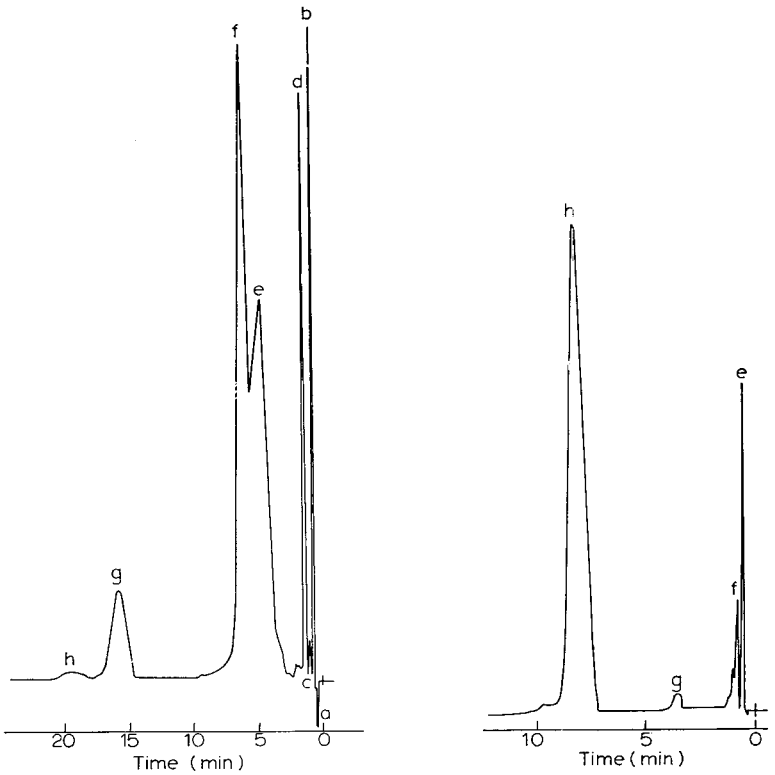


Fig. 2. Elution pattern for the trimethylstannane–pent-1-ene reaction (before decomposition). a = hydrogen; b = trimethylstannane; c = pent-1-ene; d = tetramethylstannane; e, f, h = unknown unstable compounds; g = trimethyl-*n*-amylstannane.

Fig. 3. Elution pattern for the trimethylstannane–pent-1-ene reaction (after decomposition). e = pent-1-ene; f = tetramethylstannane; g = di-*n*-butyl ether (solvent); h = trimethyl-*n*-amylstannane.

care was taken to exclude oxygen, and little tetramethylstannane was formed when trimethylstannane was heated alone. The unstable compounds were probably mixed organostannanes of the form Me_2RSnH, but although the tin–hydrogen bond reacts readily with oxygen, the decomposition of these reaction products was far more rapid than that of trimethylstannane. These unstable intermediates suggest that two mechanisms can operate in these hydrides with olefins, probably a free radical, which confirms the results of catalytic studies by NEUMANN, NIERMANN AND SOMMER[13] and FUCHS AND GILMAN[27], and a four-centre mechanism. The former reaction is favoured by higher pressures; however, further work is needed to clarify this situation.

The identities of the stable trimethylalkylstannanes formed were confirmed by:

1. Comparing their retention volumes with those of authentic samples (prepared by Grignard synthesis) and plotting a graph of log (specific retention volume) against carbon number of the chain (see Fig. 4).

2. Infra-red spectroscopy, and conventional analysis.

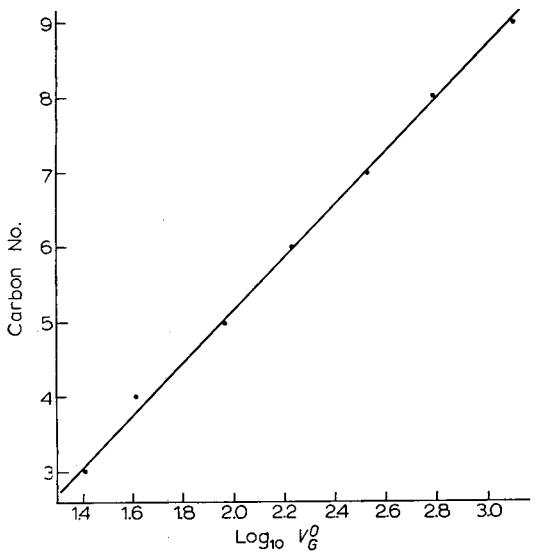


Fig. 4. Graph of log (specific retention volume) ($\log_{10}V°_G$) *versus* the number of carbon atoms in the molecule, for the trimethylalkylstannane series.

Reaction with acetylenes

VAN DER KERK AND NOLTES[18] found that organostannanes reacted far more readily with acetylenes than olefins, and this was certainly the case with trimethylstannane, the reaction being exothermic.

(*a*) *Monoacetylenes.* SMOLIN[28] studied the reaction of tri-*n*-butylstannane on acetylenes using azoisobutyronitrile as catalyst, indicating that free radicals entered into the addition reactions. By comparing the rates of reaction of trimethylstannane with olefins and acetylenes, the rate-determining step is probably the formation of radicals from the unsaturated hydrocarbons, since if the reaction was governed by formation of radicals from the stannane, the rates of reaction for the two series of compounds would be almost identical.

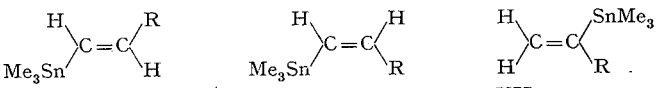
For a given triple bond, the mono- and di-addition compounds were formed, *viz.*

$$Me_3SnH + HC{\equiv}CR \longrightarrow (Me_3Sn)CH{=}CHR \quad (4)$$

$$2\ Me_3SnH + HC{\equiv}CR \longrightarrow Me_3SnCH_2{-}CH(SnMe_3)R \quad (5)$$

R = alkyl group.

The addition product formed in eqn. (4) may exist in any of the three forms below:



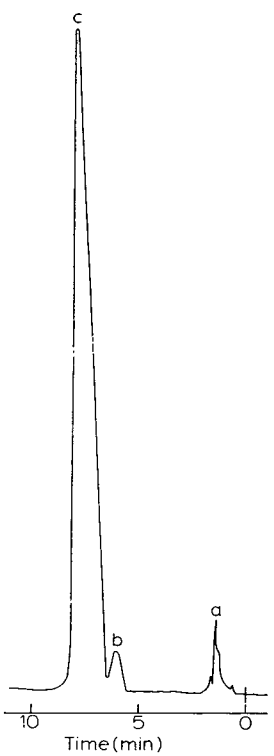
V (*trans*) VI (*cis*) VII

Chromatography of the monoaddition products formed in the first stoichiometric reaction showed that at least two components were formed, being present in the proportion of 15:1. The elution curve for the stannane–1-pentyne reaction is given in Fig. 5.

On considering the stereochemistry of the addition reaction, peak c in Fig. 5 is the product of addition at the 1-carbon atom, and is probably a mixture of V and VI. However, the *cis*-form VI would be expected to have the longer retention time, but it was impossible to resolve peak c into two peaks. Peak b must be due to structure VII; such reasoning was confirmed because for a given carbon chain, the closer towards the centre the trimethylstannane group is attached, the shorter the retention time. This was demonstrated in the series of compounds formed when trimethylstannane was added to 1-heptyne, 2-heptyne and 3-heptyne. The retention ratios of the three most stable isomers formed in these reactions over a silicone oil E-301 phase at 150° are given in Table I.

Fig. 6 shows the relationship between the log (specific retention volume) and the bonding position of the trimethylstannyl group, for the compounds in Table I. Fig. 7 shows the log (specific retention volume) and added carbon number relationship for the three series of compounds, *viz.*:

(a) 2-alkyl-1-enyl-trimethylstannanes, $H_2C{=}C(SnMe_3)R$;
(b) 1-alkyl-1-enyl-trimethylstannanes, $Me_3SnCH{=}{=}CHR$;
(c) 1,2-bis-(trimethylstannyl)-alkanes $Me_3SnCH_2CH(SnMe_3)R$.



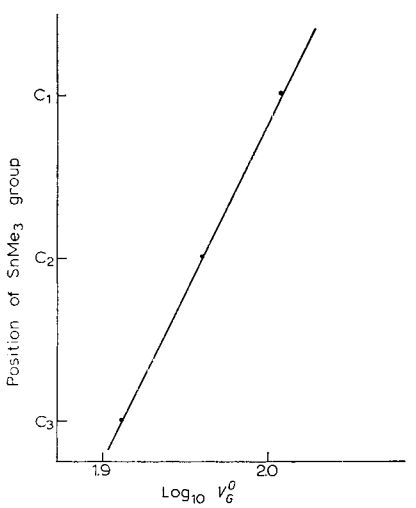
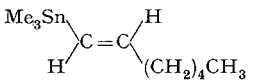
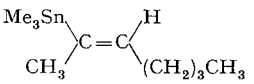
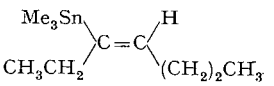


Fig. 5. Elution pattern for the trimethylstannane–1-pentyne reaction. a = tetramethylstannane; b = pent-1-enyl-(2-tin)-trimethylstannane; c = pent-1-enyl-(1-tin)-trimethylstannane.

Fig. 6. Graph of log (specific retention volume) ($\log_{10}V^{\circ}_G$) *versus* the bonding position of the trimethylstannyl group.

TABLE I

RETENTION RATIOS AND MONOADDITION STRUCTURES OF TRIMETHYLSTANNANE PLUS HEPTYNE REACTIONS

Acetylene	*1-Heptyne*	*2-Heptyne*	*3-Heptyne*
Structure of addition product			
Retention ratio	1.00	0.89 (5)	0.80

The latter have been prepared by using an excess of trimethylstannane over the stoichiometric reaction ratio with the acetylene.

$$2\ Me_3SnH + HC{\equiv}CR \longrightarrow Me_3SnCH_2{-}\underset{\displaystyle SnMe_3}{\underset{|}{C}}HR$$

Evidence for the molecular structure of the stannane-acetylene adducts was provided by the infra-red spectra. KRIEGSMANN AND PISCHTSCHAN[29] have assigned certain absorption bands in the spectra of trimethylstannyl compounds to certain atoms and groups of atoms present in the molecular structure. Table II shows the main infra-red absorption bands of the trimethylstannane–acetylene adducts together with their assigned structural vibrational frequencies.

The addition reactions of trimethylstannane and acetylenes may be followed using infra-red spectroscopy, by virtue of the strong absorption bands due to:

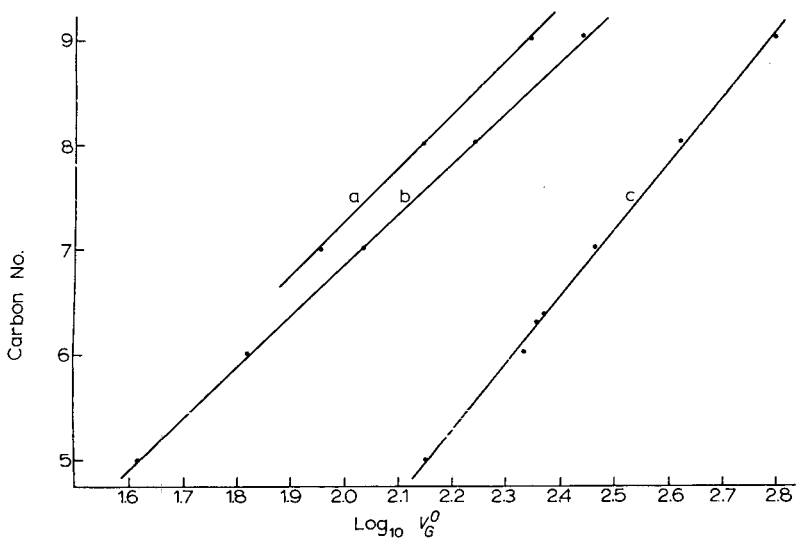


Fig. 7. Graph of log (specific retention volume) ($\log_{10}V^{\circ}_{G}$) *versus* the added carbon number for three series of compounds. a = 2-alkyl-1-enyl-trimethylstannanes; b = 1-alkyl-1-enyl-trimethylstannanes; c = 1,2-bis-(trimethylstannyl)-alkanes.

TABLE II

INFRA-RED SPECTRA OF THE TRIMETHYLSTANNANE-ACETYLENE ADDITION COMPOUNDS

Compound	*Absorption frequencies* (cm^{-1})*								
	ν_s *Sn-Me*$_3$	ν_{as} *Sn-Me*$_3$	*R Sn-Me*$_3$		δ_s *CH*$_3$		ν_{as} *CH*$_3$		δ_s *C=C*
1-Pent-1-enyl-trimethylstannane		528	715	768	1192	1381	1437	1464	1600
2-Pent-2-enyl-trimethylstannane	512	526	709	768	1191	1373	1453		1624
1-Hept-1-enyl-trimethylstannane	513	528	717	768	1192	1381	1433	1468	1600
2-Hept-2-enyl-trimethylstannane		526	709	767	1190	1378	1468		1619
3-Hept-3-enyl-trimethylstannane	514	526	708	766	1190	1380	1464		1621
1-Oct-1-enyltrimethylstannane		520	720	766	1190	1380	1437	1468	1600
4-Oct-4-enyl-trimethylstannane	513	525	709	768	1190	1379	1434	1467	1623
1-Non-1-enyl-trimethylstannane	510	527	717	764	1189	1379	1434	1466	1600
4-Non-4-enyl-trimethylstannane		522	703	758	1186	1373	1433	1459	1617
1,2-Bis-trimethylstannyl-*n*-hexane		522	710	762	1190	1381		1468	1602
1,7-Bis-trimethylstannyl-1,6-heptadiene		528	713	766	1190		1437		1600
1,9-Bis-trimethylstannyl-1,8-nonadiene		527	711	764	1183		1431	1461	1596

* ν = stretching frequency; δ = deformation frequency; R = rocking frequency; s = symmetric; as = asymmetric.

1. Tin–hydrogen bond stretching frequency (1837 cm^{-1} in trimethylstannane).
2. Olefinic carbon–carbon bond stretching frequency (1600 cm^{-1}).
3. Acetylenic carbon–hydrogen bond stretching frequency (3250 cm^{-1}).
4. Acetylenic carbon–carbon bond stretching frequency (2200–2350 cm^{-2}).

This fact is demonstrated by the infra-red spectra or trimethylstannane, 1-heptyne and trimethyl-1-(hept-1-enyl)-stannane, which are shown in Fig. 8, respectively a, b and c.

(*b*) *Diacetylenes.* The reactions of trimethylstannane with two diacetylenes were also investigated, and found to be similar to the monoacetylene reactions. The reaction with 1,6-heptadiyne yielded one diaddition product only, but that with 1,8-nonadiyne gave rise to three compounds. One is a monoaddition product, the remaining two being diaddition products. Four possible structures of the diadducts are given below:

$Me_3SnCH=CH(CH_2)_nCH=CHCnMe_3$
VIII

$Me_3SnCH_2CH(SnMe_3)(CH_2)_nC\equiv CH$
IX

$Me_3SnCH=CH(CH_2)_nC(SnMe_3)=CH_2$
X

$H_2C=C(SnMe_3)(CH_2)_nC(SnMe_3)=CH_2$
XI

The structure which would be most readily formed is VIII, and the single reaction product of the heptadiyne addition product probably has this structure where $n = 3$. Infra-red analysis showed the presence of the double bond, the trimethylstannyl group, and the complete absence of acetylenic bonds. However, such evidence does not exclude structures X and XI, but since the addition to a 2-carbon atom is so low, it is thought that the assignment of structure VIII is correct.

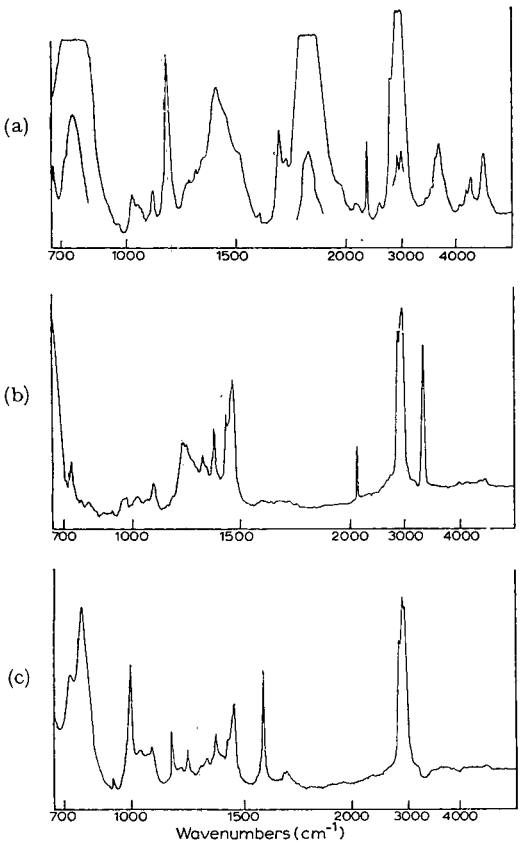


Fig. 8. Infra-red spectra of (a) trimethylstannane; (b) 1-heptyne; (c) trimethyl-1-(hept-1-enyl)-stannane.

The structure of the monoadduct formed with 1,8-nonadiyne is.

$$Me_3SnCH=CH(CH_2)_5C\equiv CH$$

The amount of monoaddition product formed, however, was extremely small and could only be detected by gas–liquid chromatography where the initial peak on an elution diagram of the reaction had a retention time almost identical to the reaction product XII with 1-nonyne.

$$Me_3SnCH=CH(CH_2)_6CH_3$$
XII

The major diaddition product was identified as the compound of structure VIII where $n = 5$, because of infra-red spectra and the points discussed with reference to 1,6-heptadiyne. The secondary product, having a shorter retention volume and comprising 5 % of the mixture, was assumed to be structure X where $n = 5$. The only

evidence for this assignment is the shorter retention time, and the relative probabilities of the formation of IX, X and XI, considering the steric hindrance involved in the three corresponding addition reactions.

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SUMMARY

Various types of organic compounds were refluxed with trimethylstannane, including halides, alcohols, mercaptans, amines, olefins and acetylenes. The products were fractionally distilled, and identified by gas chromatography, infra-red spectroscopy, etc.

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THIN-LAYER CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES OF AROMATIC ALDEHYDES AND KETONES

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INTRODUCTION

Study of the products of degradation reactions of aromatic constituents of wood has emphasized the advantages of a rapid method for the analysis and identification of aldehydes and ketones. In this field, the formation and identification of derivatives, generally 2,4-dinitrophenylhydrazones (2,4-DNPH), is necessary.

Many authors have already published papers on the qualitative analysis of carbonyl compounds by paper chromatography both on untreated and on treated paper[1–5]. The aim of the present research was to find a rapid method of separation and identification of aromatic 2,4-DNPH's. Thin-layer chromatography was investigated as this technique offers the advantages of speed of operation and has proved of value in the separation of 2,4-DNPH's[6,7].

EXPERIMENTAL AND RESULTS

Preparation of the chromatoplates

Glass plates (20 × 20 cm² and 6.5 × 20 cm²) were prepared from Silica Gel G (Merck) according to STAHL by mixing 1 part of dry powder with 2.7 parts of distilled water containing 0.5 g/l of soluble starch and 10 mg/l of sodium fluorescein. The slurry was spread on the glass plates by means of a glass rod according to the manner described by LEES AND DE MURIA[8]. The plates were dried in air for 30 min, activated at 105° for 30 min, and then allowed to cool (30 min) before applying the spots of standard solutions. The starch content of the silica gel coating only has a binding effect. A series of experiments with silica gel layers, containing different amounts of starch, showed that the separation of 2,4-DNPH's was not affected.

The presence of sodium fluorescein in the thin layer, which does not affect the R_F values of 2,4-DNPH's, enabled the substances to be located under ultraviolet light as dark spots on a yellow background.

A slurry prepared with a ratio of dry powder/water of 1:2.7 was more suitable than the usual one (1:2) for the application of a uniform layer of silica gel by the glass rod technique.

A silica gel coating of thickness of 0.5–0.6 mm was obtained by sticking three layers of adhesive tape to the opposite edges of the plate. This thickness was chosen after a series of experiments which showed that a very thin layer enhanced imperfections in the coating while a thicker one gave a poor chromatographic separation.

Variations of the thin layer thickness between 0.5 and 0.6 mm are not critical, as can be seen from Table I. The R_F values are, within certain limits, independent of the variations of layer thickness.

TABLE I

REPRODUCIBILITY OF R_F AND R_v VALUES OF ALDEHYDE 2,4-DINITROPHENYLHYDRAZONES

2,4-DNPH of	R_F		R_v	
	Average value	*S.D.*	*Average value*	*S.D.*
Vanillin	0.23	±0.013	0.71	±0.019
Veratraldehyde	0.33	±0.014	1.00	—
Anisaldehyde	0.49	±0.020	1.48	±0.012
Benzaldehyde	0.60	±0.024	1.81	±0.013

Development of chromatograms

One μl of each of the several solutions of standard 2,4-DNPH's, in chloroform or ethyl acetate, were spotted on the plates 2 cm from the base. The loading of each 2,4-DNPH spot varied from 0.1 μg to 1 μg; if the load is progressively increased beyond 1 μg, tailing rapidly becomes bad enough to spoil the chromatogram. The spotted plates were air-dried and developed, in a closed Shandon tank, at room temperature, in ethyl acetate–ligroine (75–120°) (1:2) by the ascending technique until the solvent was 14 cm above the spots (35–40 min). The spots of the 2,4-DNPH's were visible as such, but the exposure of the solvent-freed plates to ammonia vapours increased the colour intensity. The examination of the developed chromatoplates in ultraviolet light gave an even greater sensitivity, even traces of 2,4-DNPH being detectable in this way. As can be seen from the photograph in ultraviolet light, the proposed technique gives sharp round spots with a satisfactory sensitivity (0.1 μg) (Fig. 1).

Reproducibility of R_F values

The above R_F values vary with the loading of the spot, the mode of activation of the plates, the temperature and the equilibrium conditions of the chromatographic tank.

It is generally agreed that the R_F reproducibility can be considered satisfactory within the limits ± 0.05. It was determined in this case in a series of 10 experiments using plates of 6.5 × 20 cm²; these experiments were completely independent as far as the coating preparation and activation of the plates and the chromatographic tank saturation are concerned. The results are given in Table I (S.D. stands for standard deviation). It was found that better reproducibility can be obtained by recording the data in terms of the movement of a standard substance rather than of that of the solvent. The 2,4-DNPH of veratraldehyde was found useful for the purpose and the data are given in terms of R_v, defined as:

$$R_v = \frac{\text{distance in mm from the starting point to the substance}}{\text{distance in mm from the starting point to veratraldehyde 2,4-DNPH}}.$$

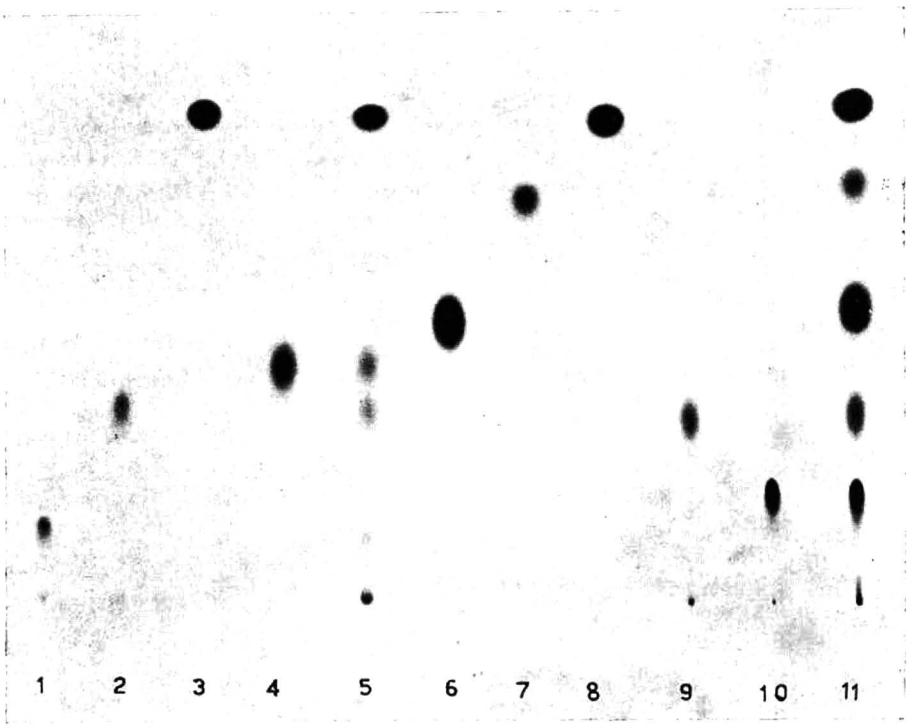

Fig. 1. Photograph in U.V. light of a chromatogram of aldehyde 2,4-dinitrophenylhydrazones. 1 = syringaldehyde; 2 = vanillin; 3 = cinnamaldehyde; 4 = *p*-hydroxybenzaldehyde; 5 = mixture of 1, 2, 3 and 4; 6 = veratraldehyde; 7 = salicylaldehyde; 8 = benzaldehyde; 9 = 2,4-dihydroxybenzaldehyde; 10 = sinapaldehyde; 11 = mixture of 6, 7, 8, 9 and 10.

The standard must be run on each plate alongside the sample to be tested.

A higher activation temperature (120°) of the thin layer or a rise of chromatographic tank temperature cause an increase of the R_F of the 2,4-DNPH spots while the R_v values are comparable to those obtained by the standard procedure; these experiments confirm the usefulness of R_v values (Table II).

TABLE II

R_F AND R_v VALUES OF ALDEHYDE 2,4-DINITROPHENYLHYDRAZONES AT DIFFERENT ACTIVATION AND ELUTION TEMPERATURES

2,4-DNPH of	R_F			R_v		
	Standard conditions	*Elution temp. 50°*	*Activation temp. 120°*	*Standard conditions*	*Elution temp. 50°*	*Activation temp. 120°*
Vanillin	0.23	0.26	0.27	0.71	0.70	0.71
Veratraldehyde	0.33	0.37	0.38	1.00	1.00	1.00
Anisaldehyde	0.49	0.55	0.56	1.48	1.47	1.46
Benzaldehyde	0.60	0.66	0.69	1.81	1.78	1.79

R_F and R_v values and chemical structure

We tried to correlate the chromatographic behaviour with chemical constitution for the 2,4-DNPH's of certain hydroxy and methoxy aldehydes and ketones, especially with respect to the number and nature of these substituent groups. In Table III the R_F and R_v values of several 2,4-DNPH's are shown. The values are the mean of six independent experiments.

TABLE III

R_F AND R_v VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF SOME AROMATIC ALDEHYDES AND KETONES

2,4-DNPH of	R_F	R_v
Benzaldehyde	0.60	1.77
Salicylaldehyde	0.48	1.41
m-Hydroxybenzaldehyde	0.32	0.94
p-Hydroxybenzaldehyde	0.30	0.88
Protocatechuic aldehyde	0.02	0.06
2,4-Dihydroxybenzaldehyde	0.23	0.68
2,5-Dihydroxybenzaldehyde	0.21	0.62
Anisaldehyde	0.48	1.41
o-Methoxybenzaldehyde	0.49	1.44
m-Methoxybenzaldehyde	0.50	1.47
Vanillin	0.23	0.68
Syringaldehyde	0.05	0.15
3-Ethoxy-4-hydroxybenzaldehyde	0.32	0.94
Isovanillin	0.23	0.68
o-Vanillin	0.32	0.94
Veratraldehyde	0.34	1.00
2,4-Dimethoxybenzaldehyde	0.41	1.21
3,5-Dimethoxybenzaldehyde	0.47	1.38
2,5-Dimethoxybenzaldehyde	0.45	1.33
2,3-Dimethoxybenzaldehyde	0.47	1.38
p-Ethoxybenzaldehyde	0.53	1.56
Acetylvanillin	0.33	0.97
4-Ethoxy-3-methoxybenzaldehyde	0.42	1.24
Cinnamaldehyde	0.60	1.77
p-Coumaraldehyde	0.33	0.97
Coniferaldehyde	0.27	0.80
Sinapaldehyde	0.15	0.44
p-Hydroxybenzylacetone	0.32	0.94
p-Methoxybenzylacetone	0.49	1.44
Acetovanillone	0.23	0.68
Acetosyringone	0.00	0.00
2,4-Dihydroxyacetophenone	0.26	0.76

From the data in Table III, it can be seen that the presence and the number of hydroxyl groups in a compound has a pronounced effect upon the R_F values. This is due to the fact that OH groups in the molecule give rise to the possibility of bonding with the absorbent silica gel coating.

The 2,4-DNPH's containing two hydroxyl groups have in general lower R_F values than the monohydroxylated ones. The *ortho* position of the two hydroxyl groups causes a strong absorption on silica gel. The *meta* and *para* positions of the two OH groups do not affect the R_F values. Methylation of hydroxyl groups causes an increase in R_F values, and in the case of the 2,4-DNPH's of monomethoxy-aldehydes

the position of the methoxy group does not affect the R_F values. For the 2,4-DNPH's of dimethoxy-aldehydes it seems that the position of the methoxy groups with respect to each other influences the R_F values.

The choice of the 2,4-DNPH of veratraldehyde as reference substance was suggested by the fact that its R_F is a limiting value between the hydroxylated 2,4-DNPH's and the non-hydroxylated ones. If $R_v > 1$, the molecule of the 2,4-DNPH does not contain free hydroxyl groups, while $R_v < 1$ would be a clear indication of the presence of hydroxyl groups.

The 2,4-DNPH of *o*-hydroxybenzaldehyde is an exception to this rule. The hydroxyl group in the *ortho* position to the aldehyde group markedly decreases the polarity; consequently, the absorption on the silica gel through the phenolic hydroxyl group is decreased by internal hydrogen bonding. In support of this, the R_F value of *o*-hydroxybenzaldehyde 2,4-DNPH is comparable with that of the 2,4-DNPH of the corresponding methylated aldehyde.

However, the phenomenon can be influenced by additional substitution in the aromatic ring, especially if this occurs *ortho* to either the hydrazone or the hydroxy group (see *o*-vanillin 2,4-DNPH). The conjugated double bond in the side chain increases the R_F values in comparison to the corresponding aldehydes without a double bond.

The present chromatographic method does not solve the problem of distinguishing between aldehydic and ketonic functions but, combined with other physical and chemical methods, it may give valuable information concerning the chemical structure of the examined compounds.

SUMMARY

Separations of 2,4-dinitrophenylhydrazones of aromatic aldehydes and ketones were achieved on thin layers of silica gel G (according to STAHL) using ethyl acetate–ligroine (1:2).

The use of a comparative method, in which veratraldehyde 2,4-dinitrophenylhydrazone is chromatographed alongside the unknown substances, gives a clear indication of the presence in the molecule of hydroxyl groups.

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THIN-LAYER CHROMATOGRAPHY ON MICROCRYSTALLINE CELLULOSE*

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INTRODUCTION

Silica gel has been widely used in the thin-layer chromatography, both qualitative and isolative, of amino acids, sugars and sugar derivatives[1,2]. The calcium sulfate binder required in this technique presents difficulties in isolative work. Paper chromatographic techniques are well established for such separations, for many of which, particularly the isolative, much time, even days, is required.

The use of cellulose spread on thin-layer plates has no doubt appealed to researchers for some time and although limited success has been recorded, the method has not been widely adopted. SCHWEIGER[3] has used "Cellulose MN 300" to separate some free sugars and alduronic acids. WOLLENWEBER[4] investigated amino acids and RANDERATH AND STRUCK[5] nucleic acid bases and nucleosides. RANDERATH[6], in comparing paper and cellulose layers, found the latter gave equal separations with more distinct zones (nucleic acid bases and nucleotides). Binders have been necessary in some cases and the layers spread were not very uniform.

In this laboratory we have prepared thin-layer plates from a commercial product known as "Avirin" ("Avicel") which is prepared by acid treatment of cellulose and represents the microcrystalline fraction of that material.

EXPERIMENTAL

Adsorbent

"Avirin" is a microcrystalline cellulose obtained from the Avicel Sales Division of American Viscose Co., Marcus Hook, Pa. Avicel is the pharmaceutical grade of the same material. We found that this grade possessed no advantage over our samples of Avirin, but either grade is satisfactory.

Preparation of chromatoplates

The microcrystalline cellulose ("Avirin" or "Avicel", 100 g) is blended in a Waring blender for 15–45 sec with 430 ml of water (the amount may vary with the particular lot) and glass plates (20 × 20 × 0.4 cm), which must be very clean, were coated with a layer 1.0 mm thick by means of a Desaga applicator. The 1.0 mm plates were found to be most advantageous for both analytical and preparative work. Plates spread only to a thickness of 0.25 mm did not show good performance. Slight differences

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with time of blending have been noted but the criterion for good smooth plates is that the blend "peaks" somewhat when poured in the applicator and the plates should be spread by drawing the applicator quite slowly, in contrast to the usual fairly rapid spreading with silica gel.

The plates are dried overnight at room temperature or for 30–60 min at 80° until the surface moisture has been released. The plates "set up" sufficiently after 30 min, and then are separated from one another, after which they may be stored for long periods of time stacked together in no special location or container much as one would store paper itself. The dried plates may be touched and written on without crumbling. They need not be desiccated or specially activated.

The compounds were dissolved in water or methanol according to their solubility and applied to the chromatoplates with a melting point capillary which had been drawn to a fine tip.

Developers

The solvent systems utilized were (A)[7] pyridine–ethyl acetate–acetic acid–water (5:5:1:3); (B) butanol–acetic acid–water (3:1:1); (C)[8] butanone–water azeotrope; (D)[9] ethyl acetate–acetic acid–formic acid–water (18:3:1:4).

Development of chromatoplates

The plates were developed in glass jars (29.5 × 27 × 10 cm) containing the solvent system to a depth of about 0.5 cm, and saturated by lining the walls with filter paper. The solvent was allowed to ascend to a height of 11–17 cm. The time of development varied from 1 to 3 h depending on the solvent. Solvent D gave the fastest and solvent B the slowest development.

Where the R_F values are low, as are most of those in Table II, a simple alternative to a commercial continuous flow apparatus may be used for better separations. A piece of Whatman No. 3 paper is attached to the top of the thin-layer plate to extend down 3 cm and is folded over the top behind the face of the plate (3–10 cm) by means of a rubber band. No problems are encountered with the cellulose crumbling under these conditions.

Spray reagents

Silver nitrate–sodium hydroxide[10] was used, followed by spraying with dilute sodium thiosulfate solution, which provided permanent zone location without heating.

In addition, the following were also used: aniline hydrogen phthalate[11], triphenyltetrazolium chloride (Ref. 1, p. 101), alkaline permanganate[12] (1 % potassium permanganate in 10 % aqueous sodium hydroxide) and 0.2 % ninhydrin in 95 % ethanol[13].

After development, the plates were dried with hot air and sprayed with the selected reagent. The free sugars and methyl glycosides were detectable, without heating, by the silver nitrate reagent while the hydroxy acids and lactones were likewise detectable, at room temperature, by the permanganate reagent. The saccharide methyl ethers were detected with the aniline phthalate or the triphenyltetrazolium reagent after heating for 5–10 min at 90–100°. The amino sugars and amino acids showed after heating for 5 min at 80° with the ninhydrin reagent. The N-

acetylamino sugars were not detected with this reagent but were shown after spraying the same plate with the silver nitrate reagent. The zones obtained were generally compact and showed little tendency to streak.

The results of the qualitative investigations are summarized in Tables I to VI.

Semimicro preparative separation

The chromatographic plate was prepared as described above. D-Galactose, 0.025 g, and D-xylose, 0.025 g, were dissolved in a small amount of water and the solution was applied to the plate by means of a sample applicator[14]. The plate was developed with solvent A. After development, the plate was dried and covered with a plastic shield to leave a 0.5-cm strip in the middle and 0.7-cm strip on each side of the plate. It was then sprayed with the silver nitrate reagent, and the zones were located and marked with a pencil. The individual bands were removed and the sugar was eluted by adding 30 ml of water. The cellulose was separated by centrifugation. This operation was repeated three times; the third eluate showed a negative reaction toward Fehling's solution. The combined eluates from each zone were evaporated under reduced pressure to 10 ml. Each fraction proved to be homogeneous by qualitative thin-layer chromatography as described above. The sugar content of a 2 ml aliquot was determined with sodium hypoiodite[15]. Found: D-galactose 0.020 g, D-xylose 0.019 g. From a 5 ml aliquot the sugars were recovered as the respective phenylosazones in good yields.

RESULTS AND DISCUSSION

"Avirin", a microcrystalline cellulose ("Avicel" is the pharmaceutical grade) produced by the American Viscose Corporation, has been found to be very useful for the thin-layer chromatography of water-soluble substances such as free sugars, glycosides, methyl ethers, hydroxy acids, lactones, amino sugars, and amino acids. We have found that Avirin cellulose in general gives more effective separations than does silica gel. For example, of the pentoses which SMITH and co-workers[2] examined, two ran the same and the third only 0.02 of an R_F unit different. In our Table I (with Solvent A) corresponding R_F values are 0.46, 0.52 and 0.59 for L-arabinose, D-xylose, and D-ribose. The R_F values for a number of monosaccharides and oligosaccharides are listed in Table I. It can be noted therein that D-glucose and D-galactose are separable in less than two hours of development time whereas on papergrams days are required to effect such a separation.

All the solvent systems we tried that have been previously reported for paper chromatography could be satisfactorily used for the thin-layer chromatography on Avirin cellulose; from these, four solvent systems were chosen as the best. The FISCHER-NEBEL[7] solvent (A) proved to be the most versatile and was found to be suitable for the separation of amino sugars and derivatives (Table II). Solvent A was also used for the preparative separation of a mixture of D-galactose and D-xylose as well as for the qualitative separation of methyl glycosides (Table III). In the latter case, the two anomeric pairs tried were separable in 2 h. Solvent C, butanone–water azeotrope, reported by SMITH and co-workers[8] as suitable for the separation of methylated sugars on paper, gave very good results on Avirin cellulose (Table IV). With these substances, an admixture of four (Table IV) was separated

TABLE I

R_F VALUES FOR SOME SACCHARIDES

Substance	R_F	
	Solvent A *	*Solvent B* **
L-Arabinose	0.46	0.31
D-Ribose	0.59	0.39
D-Xylose	0.52	0.33
D-Galactose	0.36	0.21
D-Glucose	0.39	0.25
D-Mannose	0.44	0.30
D-Fructose	—	0.29
L-Rhamnose	0.60	0.46
Cellobiose	0.25	0.13
Maltose	0.29	0.15
Maltotriose	—	0.07

* Solvent front: 13.3 cm; time: 105 min.
** Solvent front: 14.2 cm; time: 180 min.

TABLE II

R_F VALUES FOR AMINO SUGARS AND AMINO SUGAR DERIVATIVES IN SOLVENT A*

Substance	R_F
1,2-Diamino-1,2-dideoxy-D-glucitol dihydrobromide	0.11
1,2-Diamino-1,2-dideoxy-D-mannitol dihydrochloride	0.12
2-Amino-2-deoxy-D-galactose hydrochloride	0.18
2-Amino-2-deoxy-D-glucose hydrochloride	0.22
3-Amino-3-deoxy-D-mannose hydrochloride	0.24
2-Amino-2-deoxy-D-ribose hydrochloride	0.25
2-Amino-2-deoxy-D-lyxose hydrochloride	0.28
2-Amino-2-deoxy-L-xylose hydrochloride	0.31
1,2-Diacetamido-1.2-dideoxy-D-glucitol	0.38
2-Acetamido-2-deoxy-D-glucose	0.54
2-Acetamido-2-deoxy-D-glucose diethyl dithioacetal	0.85

* Solvent front: 15.5 cm; time: 115 min.

TABLE III

R_F VALUES FOR SOME METHYL GLYCOSIDES IN SOLVENT A*

Substance	R_F
Methyl β-D-arabinopyranoside	0.65
Methyl α-D-lyxopyranoside	0.75
Methyl β-D-xylopyranoside	0.70
Methyl α-D-glucopyranoside	0.57
Methyl β-D-glucopyranoside	0.61
Methyl α-D-galactopyranoside	0.54
Methyl β-D-galactopyranoside	0.56
Methyl β-D-galactofuranoside	0.72
Methyl β-cellobioside	0.44

* Solvent front: 15.6 cm; time: 120 min.

TABLE IV

R_F VALUES FOR SOME METHYL ETHERS OF SACCHARIDES (SOLVENT C*)

Substance	R_F
3-O-Methyl-D-galactose	0.02
4-O-Methyl-D-galactose**	0.02
3-O-Methyl-D-glucose	0.04
2,6-Di-O-methyl-D-galactose	0.15
2,4-Di-O-methyl-D-galactose**	0.23
2,4,6-Tri-O-methyl-D-galactopyranose**	0.48
2,3,6-Tri-O-methyl-D-mannose	0.57
2,3,6-Tri-O-methyl-D-glucose	0.60
Tetra-O-methyl-D-galactopyranose**	0.78
Tetra-O-methyl-D-fructopyranose	0.82
Tetra-O-methyl-D-glucopyranose	0.86

* Solvent front: 17.1 cm; time: 120 min.
** Separated in admixture and R_F values found to be the same as listed.

TABLE V

R_F VALUES FOR SOME HYDROXY ACIDS AND LACTONES IN SOLVENT D*

Compound	R_F
D-Arabinonic acid	0.16
Calcium D-erythronate	0.24
Glyoxylic acid	0.30
L-Erythraric [(+)-tartaric] acid	0.34
D-Arabinono-1,4-lactone	0.40
D-Erythrono-1,4-lactone	0.54
Oxalic acid	0.65

* Solvent front: 11.5 cm; time: 60 min.

TABLE VI

R_F VALUES FOR SOME AMINO ACIDS IN SOLVENT B*

Substance	R_F
Histidine	0.09
Lysine	0.10
Arginine	0.12
Serine	0.22
Threonine	0.28
Valine	0.51
Phenylalanine	0.61
Isoleucine	0.61
Leucine	0.64

* Solvent front: 16.7 cm; time: 180 min.

and the R_F values in the admixture were found to be identical with those found singly. Solvent D, ethyl acetate–acetic acid–formic acid–water (18:3:1:4), used by JONES AND WISE[9] for the separation of uronic acids on paper, has been used for the separation of hydroxy acids, lactones and aldonic acids (Table V). Paper chromatography has been an important tool in the investigation of amino acids; we found that Avirin cellulose could be used for this purpose (Table VI). The data of this table show that leucine and isoleucine are separable and that valine is separable from either.

The same spray reagents used on papergrams could be employed to detect the compounds on the thin-layer Avirin plates. Thus, alkaline silver nitrate[10] was used for the unsubstituted sugars and the methyl glycosides, aniline hydrogen phthalate[11] or triphenyltetrazolium chloride[1] for the methyl ethers of sugars, alkaline potassium permanganate[12] for acids and lactones and ninhydrin[13] for the amino sugars and amino acids. The N-acetylhexosamines were detected with the silver nitrate reagent.

A mixture of 0.025 g each of D-galactose and D-xylose was resolved on a plate with solvent A, the zones located and eluted as described in the experimental section. Estimation of the sugars with sodium hypoiodite[15] showed good recovery. From an aliquot the sugars were isolated as the respective phenylosazones in good yield.

The microcrystalline cellulose ("Avirin" or "Avicel") is much superior, in our experience, to other forms of cellulose, for thin-layer chromatography of water-soluble sugars, sugar derivatives, and amino acids. This material slurries nicely and spreads evenly. The cellulose adheres so firmly that the plates may be stacked on one another, written on, and stored without special precautions. The surface need not be specially activated. The advantages over silica gel are quite distinct and especially so with preparative separations. Their cost is about one-tenth that of silica gel G. These plates have good capacity, may be loaded with the aid of a heat gun and the material has no tendency to crumble (at 0.5 to 1.0 mm thickness), which is often the case with silica gel. When carrying out preparative work with silica gel on small amounts of natural products (especially where elution is necessary with polar solvents), one encounters contaminating colloidal silica gel and binder with the isolated zones even after extensive removal attempts.

This method of chromatography opens up the vast effective literature of papergram work while providing the ease and speed of the thin-layer technique. The zones obtained are generally very compact and show little streaking tendencies. It should readily be possible to quantitize the chromatograms by one or more of the methods established for papergrams, such as by using the proper colorimetric method for locating, removing, and eluting the zone material. The method should be well adapted for rapid undergraduate type experiments as well as for the research laboratory.

ACKNOWLEDGEMENT

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SUMMARY

"Avirin", or "Avicel", a microcrystalline form of cellulose, has been used with success in this laboratory for thin-layer chromatography. The material is employed

without a binder and has, in this laboratory, essentially completely displaced the papergram because of its speed, quality of separation, and the ease with which preparative plates may be run. In all cases tried it has been found that the papergram solvent systems and spray reagents are directly transferable to the new system, a finding which opens up the vast papergram literature to this type of thin-layer chromatography. By all comparisons, where both systems can be used, the method has proved superior, for technical and economic reasons, to that employing silica gel.

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A METHOD FOR THE THIN-LAYER CHROMATOGRAPHY OF ANALGESIC DRUGS AND RELATED COMPOUNDS IN NON-AQUEOUS SYSTEMS

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Thin-layer chromatography has been found to be eminently suited for the separation and characterization of alkaloids and other basic drugs. The extensive work in this area has been reviewed by WALDI AND GÄNSHIRT[1]. During the course of studies on the metabolism of *d*-propoxyphene, thin-layer chromatography was investigated as a possible means of separating urinary metabolites. The problems which were encountered in these studies led to the development of the method that is described in the present report.

It is essential in identification procedures involving thin-layer chromatography that the unknown be compared directly with authentic compounds. In the class of drugs under consideration, a preliminary step is often necessary to convert all samples to a common chemical form, *i.e.* either the free base or a salt. The known compounds are most often available as hydrochloride or sulfate salts, while the unknown in question is generally isolated as the free base. In the method described below, drugs may be spotted for thin-layer chromatography either in the form of the base or the salt. A single solvent is used to develop the plates in an atmosphere saturated with ammonia. Alkaloids and related synthetic compounds travel as bases regardless of whether the base or a salt of the chemical has been applied to the plate. The results are compared to those obtained on alkaline silica gel layers in the absence of ammonia.

EXPERIMENTAL

The preparative procedures that are given below generally follow the techniques that were developed by STAHL[2-5].

(*A*) *Preparation of thin-layer plates*

Glass plates (200 × 200 mm) were coated with a 250 μ layer of Silica Gel G (E. Merck, Germany) by the use of a Desaga/Brinkmann applicator (Model S II). The initial slurry was prepared by vigorously shaking 30 g of the adsorbant with 60 ml of de-ionized water. In the preparation of alkaline adsorption layers 60 ml of a 0.5 *N* LiOH solution was used instead of de-ionized water. After the silica plates had dried at room temperature, they were placed in an oven for 1 h at 110°. The plates were stored in a desiccant cabinet and were always used within the first 4 h after activation of the layer.

The compounds were spotted at 1 cm intervals in a line parallel to and 1.5 cm from the bottom of the plate. Only the center portion of the plate was used. A horizon-

tal line was drawn through the silica layer at a distance of 10 cm from the origin to establish a pre-determined solvent front.

(*B*) *Application of drugs*

Three natural and seven synthetic drugs were selected for chromatography. A sufficient amount of each compound, as the available salt, was dissolved in absolute methanol to give a concentration of 5 μg/μl. A micropipette was used to spot 25 μg of the salt of each compound at the origin. The diameter of each spot was limited to 0.5 cm.

(*C*) *Preparation of developing chamber*

Small glass tanks (215 × 215 × 115 mm) were lined with filter paper, and 110 ml of the developing solvent was poured into each chamber. At this time, care was taken to wet the filter paper liners. The internal atmosphere was allowed to equilibrate for 1 h. Twenty minutes before the chromatographic plate was to be introduced, a 30 ml beaker containing 10 ml of ammonia (28 %) was placed in one corner of the chamber. Benzene, ethyl ether and other volatile solvents readily condense on top of the evaporating ammonia solution and effectively trap the ammonia vapors. It is essential, therefore, that the tank be used shortly after the ammonia beaker is introduced. In the present study, the developing chambers were freshly prepared for each plate. Ammonia was not used when the LiOH–silica gel plates were studied.

(*D*) *Development of the thin-layer plates*

All plates were developed in an ascending manner. When the solvent reached the 10 cm mark the plate was removed. The time of solvent ascent was recorded. During the experiments the room temperature was 24 $\pm$ 0.5°. The wet plates were dried under a stream of air until the ammonia odor was gone. An iodoplatinate spray reagent[6] was used to locate the spots. The distance from the origin to the center of the spot was used in the calculation of the R_F value.

RESULTS AND DISCUSSION

In preliminary studies the salts of 10 analgesic drugs were applied to silica gel plates for chromatography. It was noted that acidic or alkaline solvent systems were required to obtain movement of the spots. Neutral organic solvents were not effective. In order to facilitate the chromatography of organic bases it has been suggested that an inorganic alkali be incorporated into the adsorption layer[5,7,8]. This modification creates an alkaline environment which increases the mobility of basic drugs by enhancing their solubility in organic solvents and by decreasing the adsorption activity of the silica layer. In this manner the desired mobility can be obtained without the addition of aqueous alkaline components to the solvent system. Amines have also been employed in the thin-layer chromatography of organic bases. WALDI *et al.*[9] have used diethylamine in a series of non-aqueous solvents to systematically analyze 54 alkaloids. The amine was incorporated in a concentration of 10 % as an integral part of the developing solvent.

The present report concerns another method for the introduction of alkalinity in adsorption chromatography without the use of aqueous solvents. If the develop-

TABLE I

THIN-LAYER CHROMATOGRAPHY OF ANALGESIC DRUGS IN AN AMMONIA ATMOSPHERE

	$R_F \times 100$** *in solvent**:													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*Mean R_F value*
d-Propoxyphene HCl	0	13	34	48	49	71	69	72	76	69	68	80	83	56.3
Cocaine HCl	0	7	17	39	51	75	72	52	68	66	63	78	79	51.3
dl-Methadone HCl	0	11	27	42	37	58	55	67	73	61	63	79	77	50.0
Anileridine di-HCl	0	0	4	8	20	37	40	27	61	61	59	77	82	36.6
Meperidine HCl	0	4	6	14	17	32	43	23	40	54	54	61	76	32.6
Ethoheptazine citrate	0	3	5	12	13	24	33	21	34	43	44	58	67	27.5
d-Methorphan HBr	0	4	4	11	11	20	32	16	24	44	45	47	64	24.8
Codeine SO_4	0	0	0	0	2	5	8	1	8	22	23	30	61	12.3
Morphine SO_4	0	0	0	0	0	0	0	0	2	9	10	12	60	7.2
Normorphine HCl	0	0	0	0	0	0	0	0	0	6	6	5	37	4.2
Mean R_F value	0	4.2	9.7	17.4	20.0	32.2	35.2	27.9	38.6	43.5	43.5	52.7	68.6	
Time for 10 cm rise (min)	17	61	31	31	39	28	36	24	30	142	129	22	39	

* Solvents: (1) petroleum ether (30–60°); (2) carbon tetrachloride; (3) isopropyl ether; (4) benzene; (5) ethylene dichloride; (6) methylene chloride; (7) chloroform; (8) ethyl ether; (9) ethyl acetate; (10) *n*-butyl alcohol; (11) isopropyl alcohol; (12) acetone; (13) methyl alcohol.

** Each figure represents the mean of 3 or more runs.

ment chamber is first saturated with ammonia vapor, single organic solvents may be used for the chromatography of organic bases and their salts. The results from this method are compared with those obtained on alkaline adsorption layers.

Table I shows the R_F values of 10 drugs in 13 organic solvents in the presence of ammonia vapor. The corresponding data for the modified adsorption layers are given in Table II. Examination of these data shows that excellent separations can be obtained without the use of water or complex solvent systems.

When the salt of a basic drug is spotted for chromatography, it is assumed that in subsequent exposure to the ammonia atmosphere the compound is converted to the free base. That this conversion occurs was verified in chromatographic studies where both the salt and the base of the 10 drugs were applied to the same plate in pairs. The resulting spots for each pair were identical in appearance and R_F value. It should be noted, however, that the initial application of the drug in the form of the base is not sufficient for movement in organic solvents. In order to effect chromatography the spots must be continually exposed to an alkaline environment during the rise of the solvent front.

The use of an ammonia development chamber eliminates the need for specially prepared plates. After development in one direction an unmodified silica gel layer can be regenerated by activation. It is then ready for use with a second solvent in two-dimensional chromatography.

The rows and columns in Tables I and II were summed and the mean R_F values calculated. Then according to the mean values, the solvents were listed in order of elutive power and the drugs in order of mobility. This presentation allows a better evaluation of the interaction of drug solubility and solvent polarity upon the R_F values.

The drugs exhibited a greater overall mobility in the ammonia atmosphere. Six or more of the compounds moved in all of the solvents with the exception of petroleum ether. In contrast, the alkaline adsorption layer was not suitable for use with solvents less polar than ethyl ether. No movement was obtained unless solvents with more elutive power were employed. The best chromatograms resulted from the use of benzene or dichloromethane in conjunction with the ammonia chamber. These two solvents gave good resolution of the test substances, and spots which were compact and well-defined. In none of the experiments, however, was streaking or diffusion a problem. By selection of the proper solvent any two of the ten drugs in this study can be separated.

In our laboratory the ammonia chamber is being employed in initial chromatographic studies on new compounds. A solubility (mobility) profile is first established using single organic solvents. If the mobility characteristics of the individual compounds are known, the proper system for chromatographic resolution can usually be devised even though the chemical may be encountered as a component of a complex mixture of drugs or metabolites. The ammonia method should also be valuable in toxicological analysis.

SUMMARY

A thin-layer chromatographic procedure is described which is applicable to natural and synthetic alkaloidal drugs. Neutral organic solvents are used to develop

TABLE II

THIN-LAYER CHROMATOGRAPHY OF ANALGESIC DRUGS ON AN ALKALINE SILICA GEL LAYER

	$R_F \times 100$** *in solvent**:													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*Mean R_F value*
d-Propoxyphene HCl	0	0	10	0	0	0	0	46	42	41	43	64	74	24.6
Cocaine HCl	0	0	4	0	0	0	0	29	38	27	36	69	73	21.2
dl-Methadone HCl	0	0	4	0	0	0	0	22	24	18	25	48	68	16.1
Anileridine di-HCl	0	0	0	0	0	0	0	19	37	39	39	62	72	20.6
Meperidine HCl	0	0	0	0	0	0	0	6	7	10	15	19	66	9.5
Ethoheptazine citrate	0	0	0	0	0	0	0	4	4	5	8	12	58	7.0
d-Methorphan HBr	0	0	0	0	0	0	0	3	3	8	9	7	54	6.5
Codeine SO_4	0	0	0	0	0	0	0	0	2	6	7	9	55	6.1
Morphine SO_4	0	0	0	0	0	0	0	0	0	4	4	4	62	5.7
Normorphine HCl	0	0	0	0	0	0	0	0	0	3	3	0	45	3.9
Mean R_F value	0	0	1.8	0	0	0	0	12.9	15.7	16.1	18.9	29.4	62.7	
Time for 10 cm rise (min)	13	30	21	25	25	18	22	16	21	110	96	14	29	

* See footnotes Table I.
** See footnotes Table I.

silica gel plates in an atmosphere of ammonia vapor. Identical results are given upon development of the plate when either the free base or a salt form is spotted for chromatography. The procedure is shown to offer more convenience and versatility than an existing method in which alkaline silica gel layers are used.

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QUANTITATIVE GRAVIMETRIC ANALYSIS OF FATTY ESTER MIXTURES BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

The use of thin-layer chromatography (TLC) for the qualitative analysis of fatty acid esters is now widespread. MANGOLD[1] separated the acetoxy-mercuri methoxy derivatives of esters into classes according to their degree of unsaturation. After decomposition of the derivatives, the compounds in each group were analysed by gas–liquid chromatography (GLC).

The ability of olefins to form labile co-ordination compounds of varying stabilities with certain metals, particularly silver, has been used as the basis for separations of unsaturated compounds and also geometrical isomers. DE VRIES achieved separations of lipid materials by column chromatography[2,3] and TLC[4] using silica impregnated with silver nitrate and BARRETT *et al.*[5] separated glycerides by TLC using the same medium. MORRIS[6] has reported the separation of higher fatty acid isomers and vinylogues by TLC using silica plates sprayed with silver nitrate or boric acid. The main advantage of these methods is that the derivatives are formed *in situ* and do not have to be decomposed after chromatography.

Techniques and apparatus have been described for preparative TLC[7,8]. Most methods for quantitative TLC[9] have involved the use of very small samples (less than 100 γ) but a semi-quantitative gravimetric method for samples of 20 to 50 mg has been reported by WILLIAMS[10].

In the present work fatty acid methyl esters were prepared from fats by the method of LUDDY[12]. With the aid of preparative TLC using Silica Gel H impregnated with silver nitrate, a sample of about 60 mg of esters was separated into groups containing saturated, mono-unsaturated, di-unsaturated and poly-unsaturated compounds. These were extracted from the silica and their proportions were determined gravimetrically. Since this work was completed, KOMAREK has reported the quantitative recovery of lipids separated by TLC[11].

EXPERIMENTAL

Preparation of esters

Methyl esters were prepared from glycerides by methanolysis with a large excess of sodium methoxide in methanol[12]. A 20% solution of the esters in ethyl ether was prepared and stored under nitrogen in a refrigerator.

Preparation of chromatoplates

Merck Silica Gel H (100 g) was shaken up in a stoppered flask for about 1 min with a 12.5 % (w/v) aqueous solution of silver nitrate (200 ml). The resulting uniform slurry was sufficient to prepare five chromatoplates 20 cm × 20 cm and 0.5 mm thick. A Shandon Unoplan Leveller and a perspex spreader were used for the preparation of the plates.

Application of sample to chromatoplate

A sample applicator has been devised which can apply about 60 mg of the esters quickly and easily in a narrow continuous streak across the chromatoplate (see Fig. 1). It was made from two thin glass plates 3.5 in. × 2.5 in. held 0.003 in. apart by a piece of copper foil 3.5 in. × 1.5 in. There was, therefore, a space 3.5 in. × 1.0 in. × 0.003 in. between the plates. The edges of the plate were ground as shown in the diagram and then polished. The applicator was filled by dipping the open edge into the sample solution and, after wipping excess from the outside, it was discharged by gently touching onto the surface of the silica gel. Two such applications, end on,

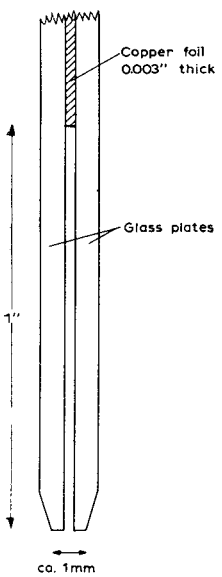

Fig. 1. Sample applicator.

with a 20 % solution of the sample gave a continuous streak of about 60 mg across the plate. The streak was applied to the plate in the position shown in Fig. 2. The plate was heated to about 70° before sample application so that the solvent evaporated quickly and the sample was contained in a narrow band.

Development of chromatoplate

A 10 % (v/v) solution of ethyl ether in 60–80° petroleum ether was used for development of the chromatogram. No equilibration period was necessary. Develop-

ment, which took about 20 min, was continued until the solvent front was about 1.5 in. from the top of the plate.

The developed plate was allowed to dry at room temperature under nitrogen, was sprayed with 0.2 % ethanolic solution of dichlorofluorescein and then was immediately examined under U.V. light. The separated compounds showed up as a series of light green bands.

Extraction and determination of the separated compounds

The bands observed corresponded to saturated, *trans*-mono-unsaturated, *cis*-mono-unsaturated, di-unsaturated and poly-unsaturated esters. The approximate positions of the bands are shown in Fig. 2. These were marked off with a fine knife blade and the silica gel in each section was transferred quantitatively to 50 ml centrifuge tubes. This was achieved by carefully scraping off with a spatula and then

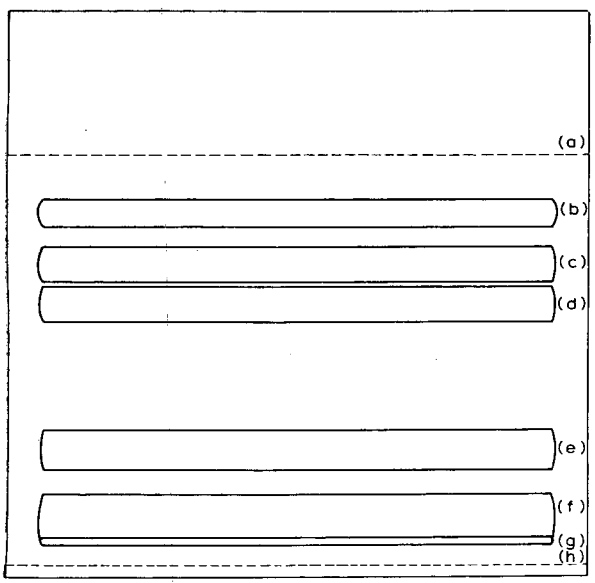

Fig. 2. Thin-layer chromatogram of fatty esters. (a) Solvent front; (b) saturated; (c) *trans*-mono-unsaturated; (d) *cis*-mono-unsaturated; (e) di-unsaturated; (f) poly-unsaturated; (g) sample streak; (h) lower edge of silica gel.

brushing off each area onto a piece of hard gloss paper before transferring to the centrifuge tube. The fractions containing *cis*- and *trans*-mono-unsaturated esters were combined. Each lot of silica gel was then extracted with three 25 ml portions of ethyl ether. The combined extracts from each section were evaporated to small bulk and transferred to 6 ml high speed centrifuge tubes. Centrifuging for 5 min at about 19.000 r.p.m. gave clear solutions which were transferred to 20 ml beakers which had been tared on a micro balance. The deposits of fine silica left in the centrifuge tubes were each extracted twice more with 4 ml portions of ether and after high speed centrifugation the extracts were added to the appropriate beakers. The combined extracts were then evaporated off under a stream of nitrogen on a steam

bath. Care was taken to prevent the beakers going dry on the steam bath. The safest procedure was to remove the bulk of the solvent on the steam bath and then blow off the last traces from the sample while it was still warm. After cooling, the beakers were re-weighed on the micro balance.

RESULTS AND DISCUSSION

A relatively simple mixture derived from linseed oil was used to test the method. The mixture was analysed by GLC alone and the results obtained are compared with values obtained from the TLC separation (Table I).

TABLE I

LINSEED OIL

Class of compound	*Percentages*	
	By TLC	*By GLC*
Saturated (C_{16} and C_{18})	10.3, 11.2, 11.2	10.2, 10.6, 9.3
Mono-unsaturated (C_{18})	20.8, 18.7, 19.8	20.3, 20.4, 20.1
Di-unsaturated (C_{18})	17.2, 16.4, 16.7	16.3, 15.1, 17.9
Tri-unsaturated (C_{18})	51.7, 53.6, 52.4	53.2, 53.9, 52.6

With mixtures of esters obtained from natural oils there is a considerable range of homologues within each group and the bands are a little wider. However, the separation between each group should be sufficient to allow for this and in work with herring oil and hardened herring oils, GLC analysis confirmed that the separated saturated, mono-unsaturated and di-unsaturated esters were free from contamination from adjacent groups. It also showed that they were not contaminated with conjugated unsaturates[4]. If any small overlaps of the groups did occur in the TLC separation, the contaminants in any group would easily be detected and estimated in the subsequent GLC analysis and allowance would be made for them.

Mono-unsaturated esters were separated into two groups containing the *cis* and *trans* isomers. In the present work they were recombined but it should be possible to extract them separately and determine their proportions.

Table II shows four sets of results obtained in the analysis of esters obtained

TABLE II

HERRING OILS

Class of compound	*Percentages*			
	Raw herring	*Hardened herring*		
		1	*2*	*3*
Saturated	24.2, 27.1, 26.4, 25.7	23.1, 23.7	37.7, 38.3	22.2, 21.2
Mono-unsaturated	60.2, 58.6, 58.1, 57.9	55.7, 55.1	50.4, 49.6	60.1, 59.0
Di-unsaturated	2.5, 4.7, 4.0, 3.1	11.1, 10.8	5.7, 6.5	14.8, 15.2
Poly-unsaturated	13.1, 9.4, 11.5, 13.3	10.0, 10.3	6.2, 5.3	2.9, 4.6

from raw herring oil. Also shown are the analyses of three samples of hardened herring oil.

Although there was no evidence of serious oxidation occurring in this work, the use of 4-methyl-2,6-di-*tert.*-butylphenol[13] would appear to be a worthwhile safeguard.

It may be possible to separate further the poly-unsaturates by using the TLC technique with a more polar mobile phase. The gravimetric method of determining compounds separated by TLC should be of use in many other problems.

SUMMARY

A thin-layer chromatographic technique, employing silica gel impregnated with silver nitrate as stationary phase, is used to separate esters into groups containing saturates, mono-unsaturates, di-unsaturates and poly-unsaturates. The proportions of these groups are determined gravimetrically and the constituent members of the first three groups can be separated, identified and determined by gas–liquid chromatography.

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THIN-LAYER CHROMATOGRAPHY OF DINITROPYRIDYL- AND NITROPYRIMIDYL-AMINO ACIDS

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Nearly all procedures employed for the determination of the N-terminal amino acids in peptides and proteins depend upon a reaction of an acylating agent with the N-terminal group; the linkage so formed must be more stable to subsequent hydrolysis of the peptide chain than the other linkages in the chain.

Research in this field began with the studies of SANGER in 1945[1], and the fluorodinitrobenzene (DNFB) method for the amino end-group analysis is now a well established procedure in protein chemistry. The weakest point in this method is that a noticeable destruction of the N-terminal dinitrophenyl (DNP)-amino acid occurs under the conditions of hydrolysis required to remove it from the protein (16–24 h with constantly boiling HCl).

In a previous paper[2], some new reagents which can be used for the identification and quantitative evaluation of N-terminal amino acids in peptides and proteins, were described.

Amongst the several investigated, 2-chloro-3,5-dinitropyridine and 2-chloro-5-nitropyrimidine are particularly useful. The most important feature of these reagents probably consists in a nucleophilic participation of the *aza-group* in the hydrolysis of the first peptide bond, permitting gentler conditions of hydrolysis (15–20 min in 6 *N* HCl with 30 % formic acid added) and the quantitative recovery of the derivative of any N-terminal amino acid, including proline and glycine[3].

Amongst the various methods by which the derivatives thus formed can be successfully separated and identified, paper chromatography has so far given very good results; some mixtures of our derivatives, however, have not yet been adequately resolved by this method. In particular, it was almost impossible to obtain a good resolution of the derivatives of aspartic and glutamic acids and of leucine and isoleucine respectively.

This difficulty can be overcome by regenerating the amino acid in the free form from the corresponding derivative in conc. NH_4OH at 110°[4]; however, the use of this indirect method requires a great deal of time.

In recent years, thin-layer chromatography[5,6] has proved exceptionally useful for the analysis of many types of compounds, providing a simple, rapid and sensitive method for the study of closely related substances, and it seems to be of particular value for compounds that are not readily separated by conventional chromatography.

In this paper we describe the separation of dinitropyridyl-amino acids (DNPyr-amino acids) and of nitropyrimidyl-amino acids (NPm-amino acids) by thin-layer chromatography on silica gel.

EXPERIMENTAL

Materials

Glass plates 20 cm × 20 cm × 0.4 cm. Silica gel G: E. Merck-A.G., Darmstadt. Spreader, chromatography tanks: Desaga GmbH, Heidelberg. Solvents: Merck, Fluka, Erba, specially prepared for chromatography.

Preparation of plates

Distilled water (50 ml) was added in a mortar to 25 g of silica gel G. The suspension was vigorously mixed for $1^1/_2$ min and the slurry formed was applied to five glass plates, using the Desaga spreader, to a thickness of 250 μ. The plates were left to dry in air overnight at room temperature, protected from dust.

Development

The technique of ascending chromatography was used throughout this work. The solvent systems[7] used were (minutes required for development in parentheses):

(A) Chloroform–methanol–acetic acid, 95:5:1 (30)

(B) *n*-Propanol-33 % ammonium hydroxide, 70:30 (120)

(C) Toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonium hydroxide[8], 100:30:60:60 (30)

(D) Benzene–pyridine–acetic acid, 80:20:2 (30)

(E) Chloroform–formic acid, 100:5 (20)

(F) Methyl ethyl ketone–pyridine–water–acetic acid, 70:15:15:2 (35)

The volume of solvent system used for development was always kept constant.

Chromatographic procedure

Amounts of 2–4 mg of the sample were dissolved in 2 ml of anhydrous methanol and 1μl, corresponding to 1–2μg, was applied with a micropipette on the starting points. The points were marked on a line at about 1.5 cm from the lower edge of the plate and with a distance of 1 cm between each point.

After evaporation of the solvent the plates were placed in the chromatography tank, previously saturated with the solvent vapours[9,10]. To ensure uniform saturation of the enclosed space, the walls of the tank were lined with filter paper soaked in solvent. The tank was filled to a depth of about 0.5 cm with the solvent system; the plates were placed on a glass support a little higher than 0.5 cm and allowed to saturate with solvent vapour for one hour. After saturation, development was initiated by increasing the level of the solvent to about 1 cm. When the solvent front had run 10 cm[11], the chromatoplates were removed and the solvent was completely evaporated in a current of hot air.

For two-dimensional chromatography the mixture was applied onto the diagonal of the plate, 3 cm from a corner. The plate was saturated in the solvent vapour for one hour and then developed with the first solvent system. The plate was withdrawn from the chromatography tank, dried for 30 min in an air current, placed in the second solvent system and developed at right angles to the first.

In order to obtain the maximum reproducibility of results, the layer must always be treated in exactly the same way before development in the second direction; in particular the conditions of intermediate drying must be kept strictly constant.

Detection method

Both DNPyr-amino acids and NPm-amino acids have high molar extinction coefficients[3], a factor which facilitates their micromanipulation and photometric estimation. DNPyr-amino acids are easily located either in natural light or in U.V. light. On the contrary the NPm-amino acids are not easily detected in natural light. We have therefore carried out the following chromatic reactions:

(1) The plates were sprayed with an aqueous solution of 1 % $KMnO_4$ and then with *N* HCl; the NPm-amino acids appeared as yellow spots on the deep pink background of the plate.

(2) The plates were sprayed with a solution of 8.1 g of $Hg_2(NO_3)_2$ in 100 ml of 0.5 *N* HNO_3 and then with 0.5 *N* HNO_3. After drying in a current of hot air, they were sprayed with an aqueous solution of $(NH_4)_2S$; the NPm-amino acids appeared as black spots on the dark background of the plate.

RESULTS AND DISCUSSION

This work deals with the chromatographic separation of DNPyr-amino acids and NPm-amino acids which can be extracted from the acid hydrolysate with organic solvents. Derivatives of arginine, cysteic acid and histidine, which are soluble in the aqueous layer, are easily separated even by conventional methods[3].

The R_F values of DNPyr- and of NPm-amino acids, in the solvent system used, are given in Tables I and II respectively.

TABLE I

EXPERIMENTAL R_F VALUES OF DNPyr-AMINO ACIDS

DNPyr-amino acids	*R_F values* (× 100) in solvent systems*					
	A	*B*	*C*	*D*	*E*	*F*
DNPyr-L-Asp	14	41	2	15	9	47
DNPyr-DL-Glu	25	42	—	25	19	57
DNPyr-DL-Ala	68	87	22	49	97	70
DNPyr-DL-Phe	79	93	30	51	103	72
DNPyr-Gly	45	78	14	30	48	65
DNPyr-DL-Ileu	91	92	38	82	130	79
DNPyr-DL-Leu	95	93	36	87	140	83
Di-DNPyr-L-Lys	86	94	41	47	74	84
DNPyr-DL-Ser	12	73	10	20	9	64
DNPyr-L-Pro	84	81	19	67	97	74
DNPyr-DL-Thr	21	74	20	25	97	63
DNPyr-DL-Val	88	92	32	75	9	78
DNPyr-NH_2	100	100	100	100	100	100
DNPyr-OH	20	87	11	13	13	83

* R_F values measured relative to DNPyr-NH_2.

The R_F values were measured relative to the corresponding dinitropyridyl- and nitropyrimidyl-amine. This enables us to obtain a more accurate and constant reproducibility of the experimental data, thereby compensating some factors relative to the development conditions and to the activation of the layer that are known[12] to have a great influence on the migration of the substances.

TABLE II

EXPERIMENTAL R_F VALUES OF NPm-AMINO ACIDS

NPm-amino acids	*R_F values* (× 100) in solvent systems*					
	A	*B*	*C*	*D*	*E*	*F*
NPm-L-Asp	18	33	13	20	16	43
NPm-DL-Glu	7	42	1	3	20	52
NPm-DL-Ala	57	73	8	64	82	78
NPm-DL-Phe	80	81	20	79	102	85
NPm-Gly	29	69	5	31	50	60
NPm-DL-Ileu	103	87	24	126	122	88
NPm-DL-Leu	94	88	23	132	119	88
Di-NPm-L-Lys	66	85	22	38	54	83
NPm-DL-Ser	6	62	4	14	13	56
NPm-L-Pro	96	77	12	94	87	70
NPm-DL-Thr	16	64	7	73	73	62
NPm-DL-Val	92	83	17	107	109	87
NPm-NH_2	100	100	100	100	100	100
NPm-OH	43	80	12	17	—	71

* R_F values measured relative to NPm-NH_2.

Considerable attention has been paid to improving the resolution of our compounds by one-dimensional chromatography. The results have shown that, among the many solvent systems tested, those described are capable of almost perfect separation, in a very short time, of the DNPyr- and NPm-amino acids investigated.

A diagram illustrating the patterns of one-dimensional separation is shown in Figs. 1 and 2.

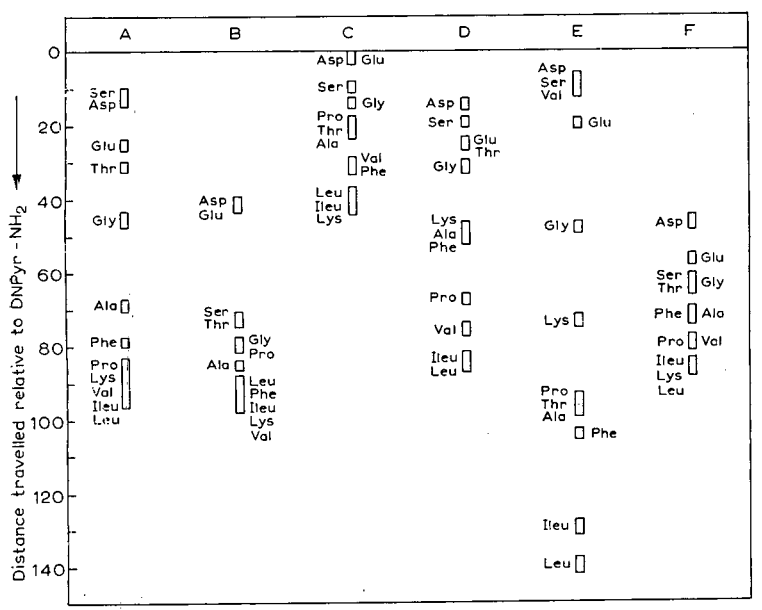

Fig. 1. Diagrammatic representation of one-dimensional separations of DNPyr-amino acids.

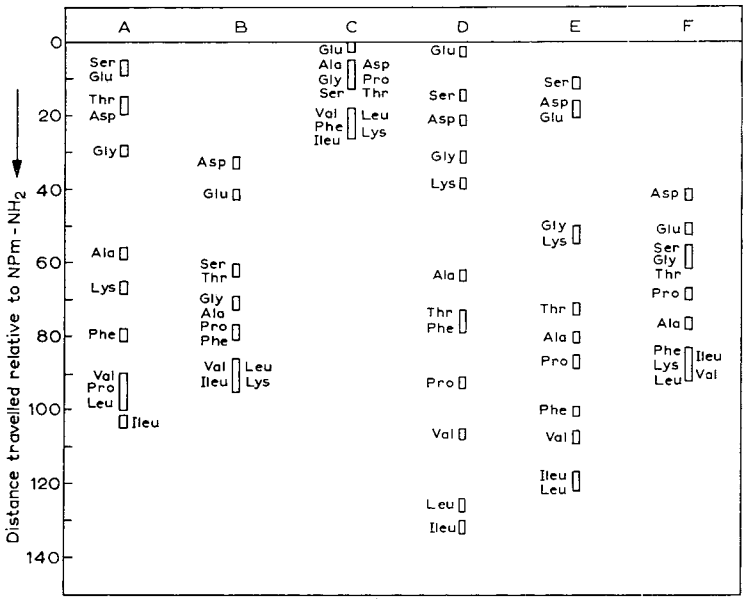

Fig. 2. Diagrammatic representation of one-dimensional separations of NPm-amino acids.

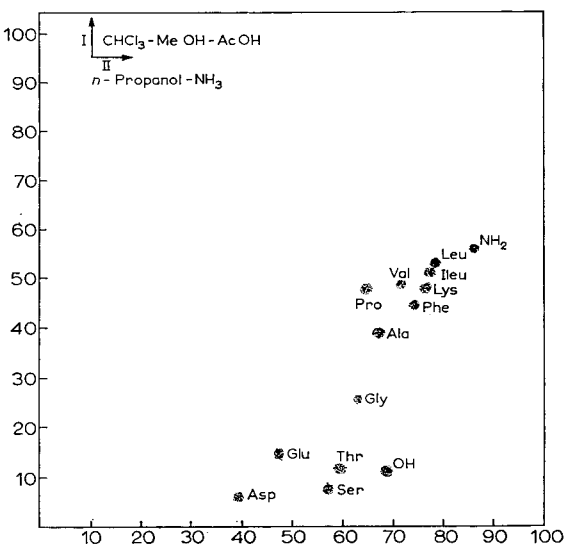

Fig. 3. Two-dimensional chromatography of a synthetic mixture of DNPyr-amino acids. Original development 10 × 10 cm.

By this method it is possible to separate the derivatives of aspartic and glutamic acid and to obtain satisfactory resolution of the derivatives of leucine and isoleucine; the latter derivatives are, however, better resolved by two-dimensional continuous chromatography[13–15].

In Figs. 3 and 4 two-dimensional separations of mixtures containing all the DNPyr- and NPm-amino acids are given.

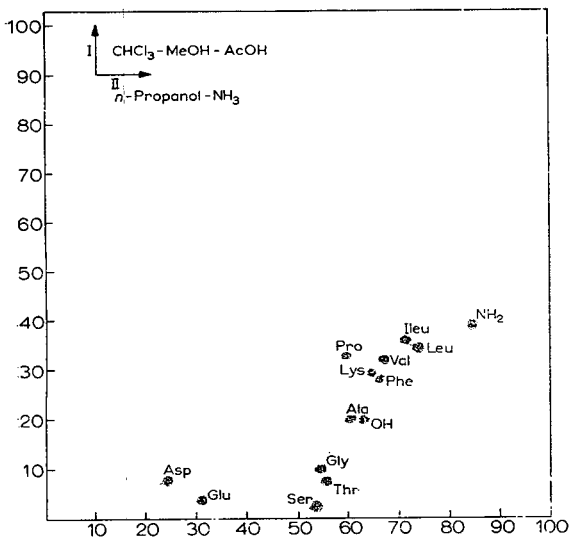

Fig. 4. Two-dimensional chromatography of a synthetic mixture of NPm-amino acids. original development 10 × 10 cm.

The preferred order of developing systems was solvent A followed by solvent B for both types of compounds. As can be seen, a good separation has been obtained both for the components of low mobility and for those of high mobility, keeping, at the same time, the distance between these two groups within reasonable limits.

SUMMARY

The separation of dinitropyridyl-amino acids and of nitropyrimidyl-amino acids by thin-layer chromatography on silica gel is described.

The advantages of this method as compared with conventional procedures are discussed.

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QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS BY THIN-LAYER CHROMATOGRAPHY*

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Thin-layer chromatography (TLC) has been extensively used in the last few years for the study of different lipid compounds. In spite of this, there are very few papers published with reference to its quantitative aspects, and furthermore, earlier papers only give very brief information about the quantitative analysis of phospholipids.

Among the various publications, the procedures used by PURDY[1], DOIZAKI[2] and AMENTA[3] seem to be, in our opinion, adequate for quantitative studies. Nevertheless, the method that we are presenting here has the advantage of being relatively quick, easy to carry out, reproducible, sensitive and accurate. The use of 1 *N* hydrochloric acid in methanol enables us to extract quantitatively, from chromatoplates, lipid compounds containing between 1 and 10 μg of phosphorus.

MATERIALS AND METHODS

Redistilled analytical solvents were used throughout.

Lipid extraction

Lipids were extracted from rat liver and brain according to FOLCH *et al.*[4] and concentrated by evaporation on a water bath at 50° under a stream of nitrogen. The final volume was calculated in such a way that approximately 4 μg of P would be present in 25 μl of lipid extract.

Thin-layer chromatography

Chromatoplates were prepared using a Desaga applicator and following the technique described by STAHL[5]. The slurry was prepared from 30 g of Silica Gel (Merck $G.F._{254}$) and 65 ml of water by shaking vigorously for 45 sec. After spreading the silica, the plates were air-dried for 20 min and activated prior to using at 110° for 30 min. They were divided into 2 cm wide lanes and the sample was applied 1.5 cm from the lower edge of the plate using a 25 μl Lange-Levy pipet. The sample was applied

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in successive small drops placed one after the other in a straight line parallel to the lower edge of the plate. This system gave a better resolution for each spot on the chromatogram.

The plates were developed in the alkaline solvent mixture described by MÜLDNER *et al.*[6], which has the advantage of giving a good separation between sphingomyelin and phosphatidyl serine. The chromatographic jar was allowed to equilibrate overnight. The chromatoplates were placed in the chamber and the solvents were allowed to ascend until they reached a distance of 1–2 cm from the top of the plate. The plates were then placed in an oven at 100° for 10 min to evaporate the remaining solvent.

Detection of the spots was carried out with iodine vapours, which according to BARRET[7] do not chemically affect the structure of the phospholipids. The area of each spot was delineated by transparency and marked on the clean side of the glass plate using a glass marker pen. The plate was left at room temperature until residual iodine had disappeared and the zone corresponding to each phospholipid, as indicated in Fig. 1, was scraped off the plate using the edge of a piece of microslide of adequate size. The silica was collected over glazed paper and carefully transferred to centrifuge tubes and eluted.

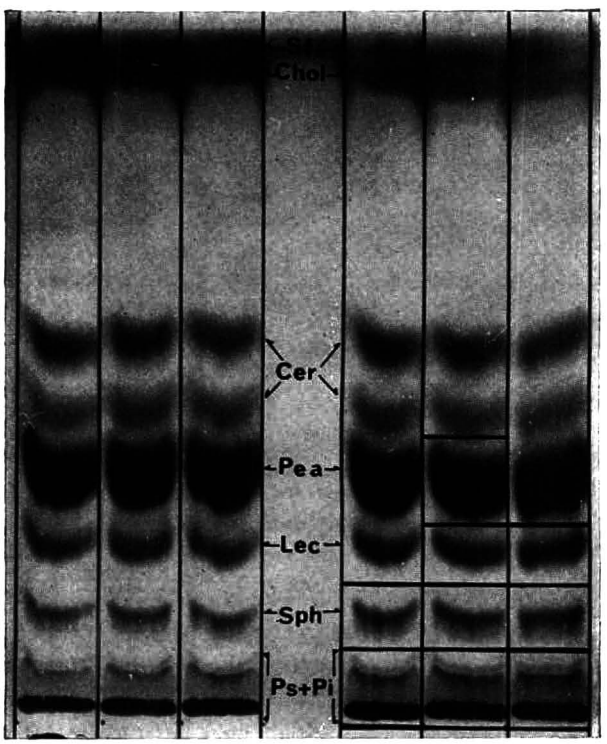

Fig. 1. TLC of rat brain lipid extract. Developing solvent: Chloroform–methanol–30% NH_3 (14:6:1). Abbreviations: Ps + Pi = phosphatidyl-serine plus phosphatidyl-inositol; Sph = sphingomyelin; Lec = lecithin; Pea = phosphatidyl-ethanolamine; Cer = cerebrosides; Chol = cholesterol; Sf = solvent front. Areas shown on the right-hand side of the chromatogram were used for the determination of P in each individual phospholipid.

Elution of the spots

The solvent used was 1 *N* HCl in methanol, which has already been employed by MARINETTI[8] for chromatography on siliconized paper. As will be seen, this solvent was the only one that gave good results among the different ones that we tried. Amounts of 4 ml of the eluting solvent were added to each centrifuge tube. The tubes were placed in a water bath at 50–60° for 15 min, stirring periodically with a glass rod. The tubes were then centrifuged for 15 min at 2000 rev./min and the supernatant was carefully decanted, the passage of the silica particles which might interfere with the colorimetric reaction being avoided. The whole procedure was repeated once more and the combined supernatants were evaporated to dryness in a phosphorus tube.

Phosphorus determination

Because of its sensitivity and accuracy, the method of CHEN *et al.*[9] was used throughout. The determination was carried out in triplicate samples. Each sample was selected in such a way that the equivalent of 1–4 μg of P would be present in each reaction tube. For this purpose, the spot given by each individual phospholipid was adjudged according to its colour intensity with the iodine vapours and when the colour was weak, two or more corresponding areas were used for each phosphorus determination (see Fig. 1). Elution and phosphorus determination were also carried out in areas of silica from the same plate in which no sample was applied (silica blank). To determine the per cent recovery obtained by our method, one of the lanes of the chromatogram was used for the determination of total phosphorus and compared with the total phosphorus in the extract.

RESULTS

In our first experiments we investigated the elution of the phospholipids from the silica. The method used by DAVISON[10] in column chromatography and later applied to TLC by the same investigator[11] did not allow us to obtain recoveries better than 80%. Although methanol had been recommended for the same purpose[12] we were not able to obtain complete recovery of the phospholipids. Chloroform–methanol mixtures in various proportions were also tried with similar results. The highest recovery obtained with these solvents was 88%. The results obtained using combinations of two solvents can be seen in Table I.

When 1 *N* HCl in methanol was first used two extractions were performed with 7 ml of this mixture on a water bath (50–60°) for 30 min. The mixture was agitated

TABLE I

RECOVERIES OBTAINED USING METHANOL AND OTHER MIXTURES SUCCESSIVELY FOR EXTRACTION*

First solvent	*Second solvent*	*Recovery*
Methanol	0.04 *N* KOH–ethanol–ether (10:7:5)	88%
Methanol	Ethanol–ether (5:5)	50%
Methanol	Ethanol–ether–chloroform (5:2:2)	60%
Methanol	Methanol–chloroform (7:3)	65%

* 10 ml of each solvent or solvent mixture were used in each extraction.

periodically, then centrifuged and the supernatant was filtered through sintered glass funnels. In view of the excellent results obtained with this method, we tried to simplify it, and finally adopted the procedure described under "Methods". Table II summarizes the results obtained using different elution techniques with the same solvent. Although the absorption given by silica is low, it should be pointed out that it is necessary to run a silica blank in all cases.

TABLE II

COMPARISON BETWEEN THE PHOSPHORUS VALUES OBTAINED IN ALIQUOTS OF RAT BRAIN LIPID EXTRACTS USING DIFFERENT ELUTION TECHNIQUES WITH 1 *N* HCl IN METHANOL

Amount of solvent (ml)	*Time of extraction (min)*	*Number of extractions*	*μg P*
7	30	2	5.65
4	30	2	5.59
7	15	2	5.53
4	15	2	5.59

In experiments carried out with different areas of silica, scraped off a plate, the readings obtained were always very close to 0.030 units of optical density. We have also carried out phosphorus determinations in the area where no phospholipids should be present (*i.e.* the area immediately below the front), with negative results.

When the mean value obtained from four experiments where the total rat brain phospholipids were eluted, chromatographed, and determined after removal from the plate was compared with the value for total rat brain phospholipids found in aliquots of total lipid extract, the recovery obtained was 96 %. A similar recovery was obtained for rat liver. To study the reproducibility of the method, nine aliquots of rat liver extract were run in separate lanes on a single plate, eluted and determined by our

TABLE III

DISTRIBUTION OF LIPID PHOSPHORUS IN RAT BRAIN*

*Phospholipid fraction***	*μg of lipid phosphorus per g of fresh tissue*										*% of lipid phosphorus*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*mean*	
TLE	2500	2112	2313	2580	2580	2220	1810	1930	2200	2250	—
Pea	1080	962	1027	915	870	710	770	850	—	898	39.8
Lec	1050	980	1015	1040	815	640	730	850	—	890	39.7
Sph	180	301	278	190	110	130	170	—	—	194	8.6
Ps + Pi	330	473	485	290	370	280	310	—	—	362	16.1

Recovery: 104.2 %***

* Results of nine different experiments.

** TLE = total lipid extract; Pea = phosphatidyl-ethanolamine; Lec = lecithin; Sph = sphingomyelin; Ps + Pi = phosphatidyl-serine plus phosphatidyl-inositol.

*** $\frac{\mu g \text{ lipid P/g fresh tissue in Pea + Lec + Sph + Ps + Pi}}{\mu g \text{ lipid P/g fresh tissue in TLE}} \times 10^2$ (recovery was calculated, using mean values).

method. The mean total lipid P value obtained was 11.3 mg per g of fresh tissue with a standard deviation of 0.3. We have also verified that the mean phosphorus value for total phospholipids eluted and determined from chromatoplates is the same as that obtained by adding the phosphorus value of the individual phospholipids eluted and quantitated in the same way. Table III shows the results for rat brain, and Table IV

TABLE IV

DISTRIBUTION OF LIPID PHOSPHORUS IN RAT LIVER*

*Phospho-lipid fraction***	*μg lipid phosphorus per g of fresh tissue*			*% of lipid phosphorus*
	1	*2*	*mean*	
TLE	9830	10420	10125	—
Pea	2342	2500	2421	24.0
Lec	5950	5890	5920	58.5
Sph	564	583	574	5.7
Ps + Pi	1117	1130	1124	11.1
Recovery: 99.3%***				

* Results of two different experiments.
** Abbreviations as in Table III.
*** Calculated as in Table III.

the results for rat liver. We have also applied this method to rat brain subcellular fractions separated by a procedure previously described[13]. The results obtained are shown in Table V.

TABLE V

DISTRIBUTION OF LIPID PHOSPHORUS IN DIFFERENT SUB-CELLULAR FRACTIONS OF RAT BRAIN*

*Phospho-lipid fraction***	*μg lipid phosphorus per g of fresh tissue*			
	Total homogenate	*Nuclear fraction*	*Mito-chondrial fraction*	*Microsomal fraction*
TLE	2376	542	692	460
Pea	996	200	236	146
Lec	1021	187	264	190
Sph	253	64	88	51
Ps + Pi	429	104	120	70
Recovery***	113%	102%	102%	100%

* Mean values of seven experiments.
** Abbreviations as in Table III.
*** Calculated as in Table III.

DISCUSSION

Although several methods have been described for the quantitative study of phospholipids separated by paper chromatography[14–16], we were interested in finding a suitable procedure for TLC. As was mentioned above, literature references to quan-

titative determination by TLC were scanty[17–20]. However, PURDY[1] had published an algebraic method for quantitative analysis of lipids separated by TLC, based on the well known linear relationship between intensity of the colour of the spot and lipid content. The main disadvantage of this method is, in our opinion, that it is difficult to use for a great number of determinations, as is the case in the study of the lipid composition of subcellular particles. Furthermore, it requires the use of lipid standards in all cases. It is a well known fact that "chromatographically pure standards" are not very pure when studied by TLC, and that lipid standards free from contaminating substances are very difficult to obtain.

The method described by DOIZAKI[2] seemed to be much easier to carry out, but it has the disadvantage of requiring a correction factor proportional to the amount employed in the determination of phosphorus (silica interferes with the colour reaction and lowers the final optical density). Furthermore, as there is no previous elution of the spots, it is difficult to carry out radioactive measurements. AMENTA[3] has recently published a very useful method for the quantitative determination of various lipids by TLC. From our point of view, the main disadvantage of this method is that it requires the use of a special type of silica gel (free of reducing compounds) and that the determination might in some cases be inaccurate, as one has to rely on the R_F values obtained in one lane developed with iodine vapour to locate the same spots in the other lanes (since residual iodine might interfere with the analysis by acting as a reducing agent if used on the other lanes).

The use of special equipment for the elution of silica, as described by MILLET[21] and GOLDRICK[12] does not seem to be necessary in our estimation because of the low standard deviation obtained by us.

The method reported in this paper uses a very simple elution procedure. Excellent recovery values can easily be obtained by extracting twice small volumes of a single solvent mixture. Of all the solvent combinations investigated, only 1 *N* HCl in methanol gave a 100 % recovery. 88 % was the highest recovery obtained using those solvents described by DAVISON[11]. Our method is also quick and very simple.

The values that we have found for phospholipids in rat liver agree with those of SCHMIDT *et al.*[22] and BIEZENSKY[23]. With reference to phospholipids in rat brain and its subcellular fractions, our data agree with those of PETERSEN AND SCHOU[24] and BRANTE[25], except for lecithin, where a difference can be observed.

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SUMMARY

A method for the quantitative analysis of phospholipids by thin-layer chromatography, based on the use of 1 *N* HCl in methanol as eluting solvent, is described.

The method is very simple, quick and reproducible. The mean value obtained for rat liver total phospholipids is 11.3, with a standard deviation of 0.3.

Data for total and individual phospholipids, determined by our method, in rat liver and brain as well as in its subcellular fractions are presented.

ADDENDUM

During the preparation of the manuscript a paper on quantitative determination of phospholipids by thin-layer chromatography by SKIPSKI *et al.*[26] has been published. The steps that we have used are similar to those which these investigators have followed. Their data agree with ours, although the method of elution and the thin-layer techniques used are different from ours.

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CHROMATOGRAPHY OF PYRAZOLE DERIVATIVES ON ACETYLATED PAPER

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INTRODUCTION

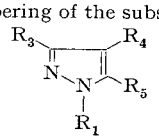
Good methods of separation and identification are absolutely necessary for chemists dealing with pyrazole ring systems. A literature search yielded only an indication of chromatographic separation of 2-substituted 5-pyrazolones[1] which differ considerably in structure by at least one substituent and in molecular weight. The common solvents used in the chromatography of the imidazoles were of no use for pyrazoles. We previously found some solvent systems suitable for the paper chromatography of pyrazole-carboxylic acids[2] (see Table I) and aminopyrazoles[3]. But for

TABLE I

CHROMATOGRAPHY OF PYRAZOLE-CARBOXYLIC ACIDS

	*Substituents**				R_F *values***
	R_1	R_3	R_4	R_5	
1	H	CH_3	H	COOH	0.22
2	CH_3	CH_3	COOH	CH_3	0.27
3	iso-C_5H_{11}	C_4H_9	H	COOH	0.79
4	C_6H_5	H	H	COOH	0.40
5	C_6H_5	H	COOH	H	0.33
6	H	C_6H_5	H	COOH	0.35
7	C_6H_5	CH_3	H	COOH	0.47
8	C_6H_5	COOH	H	Cl	0.48

* Numbering of the substituents in the pyrazole ring for this table and all subsequent tables is as follows:



** Solvent system: *tert.*-butanol–petroleum ether–2 *N* ammonia (25:5:2).

many pyrazoles, because of their low solubility in water, or because of difficulty of detecting chromatographic quantities, a paper chromatographic method could not be developed.

Pyrazoles that do not have an acidic function were separated and identified in almost all cases by thin-layer chromatography on Al_2O_3[3,4] (see Table II). But in

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this case, condensed systems or those with a strongly polar group could not be separated because of very low R_F values.

In the present work, we found that chromatography on acetylated paper gives a general method for the separation and identification of pyrazole compounds. This method can be used to control the direction and course of relatively complicated reactions. Acetylated paper permits the chromatography of significantly more substance than ordinary paper. In our case, with the same paper acetylated, the optimal quantity rises from 15–30 μg to 50–100 μg, making the identification of impurities with small R_F differences easier.

TABLE II

THIN-LAYER CHROMATOGRAPHY OF PYRAZOLE DERIVATIVES

	Substituent				*R_F values**		
	R_1	R_3	R_4	R_5	*1*	*2*	*3*
1	CH_3	CH_3	H	H	0.52	0.48	—
2	CH_3	H	H	CH_3	—	0.41	—
3	H	CH_3	H	CH_3	0.05	0.03	—
4	C_3H_7	CH_3	H	CH_3	0.55	0.49	—
5	C_5H_{11}	CH_3	H	CH_3	0.61	0.59	—
6	C_8H_{17}	CH_3	H	CH_3	0.67	0.66	—
7	$C_{16}H_{33}$	CH_3	H	CH_3	0.73	0.69	—
8	C_6H_5	H	H	H	0.69	0.71	—
9	C_6H_5	CH_3	H	CH_3	0.59	0.64	—
10	CH_2–C_6H_5	CH_3	H	CH_3	0.78	0.75	—
11	H	CH_3	NO	CH_3	0.01	0.03	—
12	CH_2–C_6H_5	CH_3	NO	CH_3	0.52	0.43	—
13	C_5H_{11}	CH_3	NO	CH_3	0.53	—	—
14	H	CH_3	$COCH_3$	CH_3	0.00	—	0.04
15	H	CH_3	COC_6H_5	CH_3	0.03	—	0.03
16	CH_3	CH_3	$COCH_3$	CH_3	0.27	—	0.49
17	CH_3	CH_3	COC_3H_7	CH_3	0.35	—	0.54
18	C_6H_5	CH_3	$COCH_3$	CH_3	0.43	—	0.66
19	C_6H_5	CH_3	H	H	0.70	—	0.84
20	C_5H_{11}	CH_3	COC_6H_5	CH_3	0.44	—	0.73

* Solvent systems: (1) benzene–chloroform (1:1); (2) petroleum ether–chloroform (1:1); (3) petroleum ether–chloroform (1:5).

EXPERIMENTAL

Chromatographic paper "Fast", from Volodarskii Factory (Leningrad), corresponding approximately to Whatman No. 1, was acetylated by ZIJP'S[5]* method. Our technique of chromatographic separation was based on the method described by KŐSTIŘ AND SLAVIK[6]. As in ordinary paper chromatography[2], we worked with a battery of test tubes 250 × 25 mm. Only in certain cases, when the R_F values of the compounds to be separated were very close to each other or when we had a mixture of unknown substances to identify, did we use paper strips 35 cm long. The solvents used were the same as in ref. 6.

* In the abstract there is a misprint: 100 ml of toluene instead of 1000 ml in the original paper.

On the bottom of the test tubes we placed some solvent of the stationary phase ($CHCl_3$, C_6H_5Cl) and after spotting the substance on the starting point of the acetylated paper strip, we let it hang in the closed test tube, without touching the surface of the solvent, for 10–15 min, and then transferred it to another test tube containing the development solvent (moving phase), generally 80 % ethyl alcohol. In this test tube we attached to the cork stopper a little piece of cotton-wool, and immediately before beginning the development this was wetted with a few drops of the stationary phase. Development for 10–12 cm takes 15–20 min, and the complete operation takes 30–50 min. If *n*-octanol, which solves cellulose acetate but does not dissolve it, is employed as stationary solvent the acetylated paper strip is immersed in a 5 % solution of *n*-octanol in methanol for 30 min and then dried in air for 50–60 min[6].

With a long strip the technique is as follows: 50 ml of stationary solvent and an empty Petri dish were placed on the bottom of a cylindrical chromatographic chamber 45–50 cm high. The strip is spotted at the starting points, dried and hung in the solvent vapour for 20–25 min. 30 ml of development solvent is poured into the Petri dish and the chromatograph is run until the solvent front rises approx. 30 cm. This takes, at 18–20°, with ethyl alcohol as development solvent, 2.5–3 h, a relatively short time as compared with ordinary paper chromatography. The sample should be dissolved in alcohol, methanol or other solvent inert to cellulose acetate[6].

Spots were identified with the help of U.V. light from an "Ultraquimiscope UI-1" source, with filters "UFS-1" (U.S.S.R.) or with iodine vapour[7,8] (from crystals in a closed chamber) or chlorine[8]. As the acetylated paper is hydrophobic and soluble in many organic solvents, the identification of spots with reagent solutions is limited compared with ordinary filter paper. Some coloured substances, dyes, etc., are visible on the paper and need no special identification method.

RESULTS

The results given in Tables III-V clearly show the versatility of the method for different pyrazole systems, from alkyl-aryl-pyrazoles (Table III) to azopyrazolone dyes (Table IV) and bi-heterocyclic pyrazole compounds with fused rings (Table V).

In Tables III and IV we see the dependence of the R_F value on the nature and position of the substituents. For derivatives of 1-phenyl-3-methylpyrazole, with the same solvent system ($CHCl_3$–80 % C_2H_5OH), the effect of the substituent in position 5 of the pyrazole ring on the R_F value is as follows: CH_3 R_F 0.18 (Table III, No. 4); NH_2 R_F 0.26 (Table III, No. 13); OH R_F 0.45 (Table IV, No. 2); COOH R_F 0.54 (Table IV, No. 22); SH R_F 0.70 (Table IV, No. 6). Such a succession and approximately similar sorts of R_F values are seen for other 1-phenylpyrazoles with other substituents, and other solvent systems.

A CH_3 group on position 3 of the pyrazole ring has in general a weak influence on the R_F value of the compound; but this influence depends on the presence of other substituents. As can be seen from Table III (No. 12 and 13) it is insignificant for 1-phenyl-5-aminopyrazoles, but increases significantly for 1-phenyl-5-pyrazolones (Table IV, No. 1 and 2), for 1-phenyl-5-carboxylic acids (Table IV, No. 21 and 22) and 1-phenyl-5-chloropyrazoles (Table III, No. 5 and 6).

For the 5-pyrazolones this may be explained by the greater influence of the

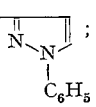
TABLE III

CHROMATOGRAPHY OF PYRAZOLE DERIVATIVES ON ACETYLATED PAPER

	Substituent				*R_F values**	
	R_1	R_3	R_4	R_5	1	2
1	H	CH_3	H	CH_3	0.60	0.60
2	H	C_6H_5	H	C_6H_5	—	0.17
3	CH_3	CH_3	H	CH_3	0.70	0.90
4	C_6H_5	CH_3	H	CH_3	0.18	0.16
5	C_6H_5	H	H	Cl	—	0.24
6	C_6H_5	CH_3	H	Cl	0.18	0.07
7	C_6H_5	Cl	H	H	0.20	0.15
8	H	C_6H_5	H	NH_2	0.53	0.35
9	iso-C_3H_7	CH_3	H	NH_2	—	0.80
10	iso-C_3H_7	*p*-$C_6H_4NH_2$	H	NH_2	0.30	0.10
11	CH_3	*p*-$C_6H_4NH_2$	H	NH_2	0.30	0.20
12	C_6H_5	H	H	NH_2	0.26	0.16
13	C_6H_5	CH_3	H	NH_2	0.26	0.20
14	C_6H_5	H	NH_2	H	0.45	0.15
15	C_6H_5	NH_2	H	H	0.21	0.18
16	CH_2-C_6H_5	CH_3	H	NH_2	0.70	0.76
17	CH_3	CH_3	NO_2	CH_3	0.40	0.30
18	CH_3	CH_3	SCl	CH_3	0.50	0.33
19	iso-C_3H_7	CH_3	NO	CH_3	0.64	0.28
20	CH_2CH_2CN	C_6H_5	H	C_6H_5	—	0.23
21	C_6H_5	H	OCH_3	H	0.21	—
22	C_6H_5	H	HgCl	H	0.24	—
23	C_6H_5	H	HgBr	H	0.20	—
24	*p*-$NO_2 \cdot C_6H_4$	CH_3	NO_2	Cl	0.10	0.10
25	*p*-$SO_3H \cdot C_6H_4$	H	H	C_6H_5	0.20	0.17
26	NO**	CH_3	H	C_6H_5	0.30	0.22
27	$COCH=CHCO_2H$	CH_3	H	α-furyl	0.68	0.60
28	$COCH=CHCO_2H$	C_6H_5	H	α-furyl	0.20	0.20
29	iso-C_3H_7	CH_3	X***	OH	0.49	0.44
30	iso-C_3H_7	C_6H_5	X	OH	0.30	0.25
31	CH_2CH_2CN	CH_3	X	OH	0.00	0.15
32	$CH_2CH_2C_6H_5$	CH_3	X	OH	0.23	0.20
33	C_6H_5	CH_3	CH_2OH	Cl	0.19	—
34	C_6H_5	CH_3	H	Y***	0.63	0.79
35	C_6H_5	CH_3	H	Z***	0.56	0.40
36	C_6H_5	H	H	Y	—	0.30
37	C_6H_5	H	H	Z	—	0.20
38	C_6H_5	H	H	Y	—	0.60
39	C_6H_5	H	Y	H	—	0.59
40	C_6H_5	Z	H	H	—	0.10
41	C_6H_5	Y	H	H	—	0.60
42	C_6H_5	CH_3	H	Y	—	0.40

* Solvent systems: (1) chloroform (stationary phase)–80% ethanol; (2) chlorobenzene–80% ethanol.

** Pyrazoline ring.

*** X =  ;

Y = $-HN \cdot CO \cdot CH=C(OH) \cdot CH_3$;
Z = $-HN \cdot C(CH_3)=CH \cdot CO_2C_2H_5$.

TABLE IV

CHROMATOGRAPHY OF PYRAZOLE DERIVATIVES ON ACETYLATED PAPER

	Substituent				*R_F values**			
	R_1	R_3	R_4	R_5	*1*	*2*	*3*	*4*
1	C_6H_5	H	H	OH	0.57	0.40	0.60	—
2	C_6H_5	CH_3	H	OH	0.45	0.20	—	—
3	C_6H_5	H	OH	H	0.40	0.17	0.11	—
4	C_6H_5	OH	H	H	0.40	0.30	0.25	—
5	C_6H_5	OH	H	C_6H_5	0.40	—	—	—
6	C_6H_5	CH_3	H	SH	0.70	—	—	—
7	CH_3	CH_3	$COCH_3$	CH_3	—	0.68	—	—
8	CH_3	CH_3	COC_3H_7	CH_3	0.69	—	—	—
9	$CH_2 \cdot C_6H_5$	CH_3	COC_6H_5	CH_3	—	0.70	—	—
10	C_6H_5	CH_3	$COCH_3$	CH_3	0.50	0.30	—	—
11	C_6H_5	CH_3	COC_6H_5	CH_3	0.80	0.80	—	—
12	C_6H_5	H	$COCH_3$	Cl	—	0.24	—	—
13	C_6H_5	CH_3	$COCH_3$	Cl	0.20	0.20	0.20	—
14	C_6H_5	Cl	$COCH_3$	H	0.34	0.80	0.18	—
15	H	CH_3	$COCH_3$	CH_3	0.20	0.18	—	—
16	$COCH_3$	C_6H_5	H	C_6H_5	0.87	0.30	—	0.40
17	COC_6H_5	CH_3	H	CH_3	0.50	—	—	—
18	H	CH_3	H	COOH	—	0.30	0.50	0.40
19	H	C_6H_5	H	COOH	0.30	—	—	—
20	C_5H_{11}	C_4H_9	H	COOH	0.70	0.32	0.70	0.20
21	C_6H_5	H	H	COOH	0.40	0.20	0.36	0.30
22	C_6H_5	CH_3	H	COOH	0.54	0.36	0.50	0.45
23	C_6H_5	COOH	H	Cl	0.50	0.20	0.30	0.28
24	C_6H_5	CH_3	COOH	Cl	0.27	—	—	—
25	$CH_2 \cdot C_6H_5$	CH_3	H	COOH	0.70	—	—	—

* Solvent systems (stationary phase): (1) chloroform; (2) chlorobenzene; (3) isoamyl chloride; (4) benzyl chloride. Development solvent (in all cases): 80% ethyl alcohol.

inductive effect of the electron-donor group CH_3, as the influence of this group on the tautomeric equilibrium between "oxo" and "oxi" forms[9] has already been demonstrated. The content of oxo-form in the equilibrium, already great for 1-phenyl-5-pyrazolone in $CHCl_3$, rises significantly with the introduction of a CH_3 group in position 3, and this considerably modifies the R_F. The NH_2 groups, on the contrary, always exist in amino form[10] and are not influenced significantly by a CH_3 group.

In the case of carboxylic acids and chloro-derivatives, which have a strong inductive $-I$ effect, we expect a rise in polarity on the introduction of a CH_3 group (positive $+I$ effect) in position 3, with a consequent modification of R_F.

For a substituent in position 1 or 3 we found an interesting rule: when substituting an alkyl (or benzyl) for a phenyl substituent, we observed a fall in R_F value in the solvent systems $CHCl_3$–80% C_2H_5OH and C_6H_5Cl–80% C_2H_5OH. As examples see Table III, No. 1 and 2, No. 3 and 4, No. 9, 13 and 16, No. 27 and 28, No. 29 and 30, and (weaker) Table IV, No. 22 and 25. So, if all the substituents on a pyrazole ring are the same, except one in position 1 or 3, the R_F values will show, with some degree of reliability, whether the substituent is an alkyl, aralkyl or a phenyl.

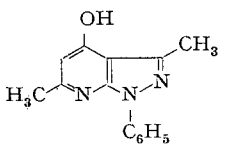
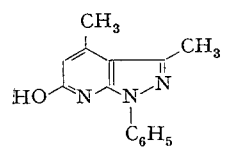
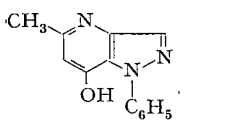
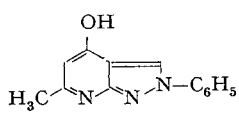
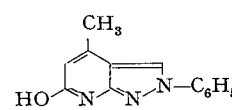
While investigating halogenated compounds that dissolve cellulose acetate as stationary phase solvents, and consequently solve acetylated paper, we came to

the conclusion that there are at least two groups of solvents that can be distinguished by the R_F of some of the pyrazole compounds. One group includes chlorobenzene and benzyl chloride, the other, chloroform and isopropyl chloride. The R_F of many substances is lower in the system chlorobenzene–80 % ethyl alcohol than in the system chloroform–80 % ethyl alcohol. This rule is valid for compounds having the most diverse functions and we could not explain it. We thought that the determining factor might be the dipole moment of the solvent, but we had to abandon this hypothesis because the μ for isopropyl chloride (1.93 in benzene) is nearer to the μ for benzyl chloride (1.85) than to the μ for chloroform (1.15 in benzene), and the difference between the μ for chlorobenzene (1.56) and benzyl chloride is greater than the difference between the μ for isopropyl chloride and benzyl chloride[11], thus invalidating the hypothesis.

Other alcohols (*n*- and iso-propanol, *n*- and *tert.*-butanol) were tried as development solvents, but the change in R_F of the compounds does not justify their use instead of ethyl alcohol.

TABLE V

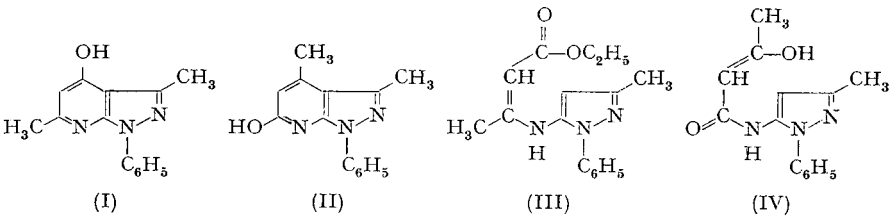
CHROMATOGRAPHY OF POLYCYCLIC COMPOUNDS OF PYRAZOLE WITH FUSED RINGS ON ACETYLATED PAPER

	Compound	*R_F values**	
		1	*2*
1		0.75	0.68
2		0.40	0.30
3		—	0.26
4		—	0.80
5		—	0.60

* Solvent systems: (1) chloroform–80 % ethanol; (2) benzyl chloride–80 % ethanol.

In the chromatography of ketones it is common practice to convert them to dinitrophenylhydrazine derivatives; as the dinitrophenylhydrazones are all coloured, there is no need for identification reagents. In our case (Table IV) the dinitrophenylhydrazones gave very long spots and an R_F value would have no meaning.

On the basis of our results we employed chromatography on acetylated paper to study the synthesis of polycyclic compounds with fused rings (Table V). Under different conditions[12] we attempted to synthesise the isomeric 1-phenyl-3,6-dimethyl-4-hydroxypyrazolo(3,4-*b*)pyridine (I) and 1-phenyl-3,4-dimethyl-6-hydroxypyrazolo-(3,4-*b*)-pyridine (II), from the intermediate compounds (III) and (IV), respectively.



(I) (II) (III) (IV)

In contradiction to the opinion generally accepted after the work of BÜLOW[13], we found that the reaction of 1-phenyl-3-methyl-5-aminopyrazole with acetoacetic ester in boiling acetic acid gives compound (II), and not even traces of (I) were found. Both intermediate products (III) and (IV), in boiling acetic acid, always gave the same pyrazolo(3,4-*b*)pyridine (II). Compound (III) is probably converted to (IV) and from this the pyridine ring is formed in the usual way, to give (II). To confirm this hypothesis we boiled 1 g (III) for 15 min in glacial acetic acid and precipitated the compounds formed with water (all were very insoluble, with the exception of the 1-phenyl-3-methyl-5-aminopyrazole). A sample of the mixed solids dissolved in methanol and chromatographed showed the presence of 3 compounds: a little of the original compound (III), the pyrazolo(3,4-*b*)pyridine (II) and traces of the 1-phenyl-3-methyl-5-aminopyrazole. The R_F values of all of these compounds are sufficiently different from each other and from the R_F of (I), in the solvent system $CHCl_3$–80 % C_2H_5OH, to leave no doubt about their identity (see Table V, No. 1 and 2; Table III, No. 13, 34 and 35). This proves that under these conditions compound (I) is not formed; in the first stage, probably compound (IV) is formed, but it could not be detected because the cyclisation to (II) is very fast.

ACKNOWLEDGEMENT

We thank Miss T. GULIAEVA for her assistance in the experimental work.

SUMMARY

The chromatography of pyrazoles on acetylated paper is described. An attempt is made to correlate some of the chromatographic data with the structure of compounds. The influence of the inductive effect of the substituents is clearly seen, especially for 1-phenyl-3-methyl-5-x-pyrazoles. The R_F of an alkyl derivative (the

other substituents maintained) is greater than the R_F of the corresponding aryl substituted pyrazole.

In a condensed system, such as pyrazolo-pyridine, the α-pyridone can easily be distinguished from the γ-pyridone.

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PAPER ELECTROPHORESIS OF STEROIDS IN BORATE BUFFERS

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INTRODUCTION

Paper electrophoresis in borate buffers has been used extensively for the separation and identification of polyhydroxylated carbohydrates[1–3]. Carbohydrates, in which the hydroxyl groups are vicinal and *cis*-oriented, form negatively charged complexes with borate that migrate toward the anode. Complex formation between borate and steroidal *cis*-dihydroxyl groups is also known to occur. Paper chromatography in the presence of borate has been used to indicate the configuration of a number of vicinal hydroxyl groups of steroids[4,5]. Borate derivatives of two 16α,17α-dihydroxy steroids have been prepared and characterized by LEESON *et al.*[6]. BULASCHENKO *et al.*[7], in a study of steroid metabolism, employed paper electrophoresis in borate buffer for the recognition of steroids containing hydroxyl groups of C-20 and C-21. The application of paper electrophoresis to C-16,17-dioxygenated steroids, as well as certain other polyhydroxylated steroids, is described in this paper.

EXPERIMENTAL

Borate buffers were prepared by adjusting a 0.3 *M* boric acid solution to the desired pH with 10 *N* sodium hydroxide. The solution was then diluted with an equal volume of reagent-grade methanol.

Electrophoresis was performed on Whatman 3 MM paper that had been saturated with the electrolyte, blotted, and placed in a horizontal electrophoresis migration chamber*. The ends of the paper were dipped into the borate solution contained in the electrode vessels. The paper and the electrolyte were allowed to equilibrate for a period of 30–60 min prior to application of the steroids (10–50 μg) in methanolic solution. In most instances electrophoresis was carried out at room temperature for 5 h at 500 V (10.9 V/cm).

9α-Fluorocortisol (XVI)**, which does not form a charged complex with borate, was included in each experiment to serve as an indicator of electro-osmotic flow. The position of each steroid was measured relative to that of XVI, and the anodic migration (M_s) was expressed as a fraction of the distance travelled by the borate-complexing steroid, 9α-fluoro-16α-hydroxycortisol (XVII).

* Model E800-2B, Research Specialities Co., Richmond, Calif.
** Refer to tables for identy of steroids.

The steroids were detected by methods commonly used in paper chromatographic analysis[8].

RESULTS AND DISCUSSION

Effect of pH

The rate of anodic migration of steroids in borate buffers was dependent on pH (Fig. 1). The mobility of a 16α,17α-dihydroxy-20-ketosteroid (XVII) increased sharply from pH 6.1 to about pH 8.5, and then decreased slightly. The migration rate of other steroids (I, IV, XXV) was less susceptible to changes in the pH 6–8 range, but increased rapidly under more alkaline conditions. All complexing steroids streaked at pH values below 7.0.

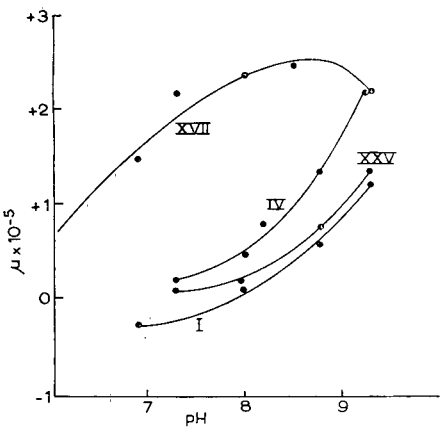

Fig. 1. Effect of pH upon electrophoretic migration. μ = Electrophoretic migration = $\frac{\text{velocity in cm/sec}}{\text{voltage/cm}}$. Negative value denotes cathodic migration. I = 16α-hydroxyandrost-4-ene-3,17-dione; IV = 16α,17α-dihydroxy-17β-methylestr-4-en-3-one; XVII = 9α-fluoro-11β,16α,17α,21-tetrahydroxypregn-4-ene-3,20-dione; XXV = 17α,20β,21-trihydroxypregn-4-en-3-one.

C-16,17-Dioxygenated steroids (cf. Table I)

The anodic migration of 16α,17α-dihydroxysteroids could be prevented by acetylation of one of the *cis* hydroxyl groups, *e.g.* compounds VIII and IX. At high pH values however, some hydrolysis of the ester probably occurred with subsequent delayed migration of the free steroid.

16α-Hydroxylated steroids that contain a 17β-hydroxy group (II, III), or that lack any oxygen function at C-17 (VI, X) displayed a slight cathodic migration, for which we can offer no explanation. A 16β,17α-dihydroxysteroid (XIII), which would not be expected to complex with borate, exhibited two anodic migrating zones, perhaps caused by isomerization to compounds XII, XIX, and/or XX. Such isomerization has been shown to occur when 16β-acetoxy-17α,21-dihydroxypregn-4-ene-3,20-dione was subjected to basic conditions[9].

The presence of a C-20 ketone apparently contributes to the rate of anodic migration of 16α,17α-dihydroxysteroids, since blockage of the C-20 ketone (XV), or its absence (IV), resulted in a lowered migration rate. Moreover, compound XVIII,

TABLE I

ANODIC MIGRATION OF C-16- AND C-17-OXYGENATED STEROIDS

Steroid		M_s[a]	
		pH 8.5	*pH 9.3*
I	16α-Hydroxyandrost-4-ene-3,17-dione	0.28	0.50
II	16α,17β-Dihydroxyandrost-4-en-3-one		[b]
III	16α,17β-Dihydroxy-17α-methylestr-4-en-3-one		[b]
IV	16α,17α-Dihydroxy-17β-methylestr-4-en-3-one	0.55	0.91
V	16β-17β-Dihydroxy-16α-methylestr-4-en-3-one		0.74
VI	16α-Hydroxypregn-4-ene-3,20-dione		[b]
VII	17α-Hydroxypregn-4-ene-3,20-dione	0.00	
VIII	16α,17α-Dihydroxypregn-4-ene-3,20-dione	1.00	1.09
IX	16α-Acetoxy-17α-hydroxypregn-4-ene-3,20-dione	0.00	0.07
X	16α,21-Dihydroxypregn-4-ene-3,20-dione		[b]
XI	17α,21-Dihydroxypregn-4-ene-3,20-dione		0.00
XII	16α,17α,21-Trihydroxypregn-4-ene-3,20-dione	1.00	1.00
XIII	16β,17α,21-Trihydroxypregn-4-ene-3,20-dione		0.59 and 1.00
XIV	20-Ethylenedioxy-11β,17α,21-trihydroxypregn-4-en-3-one		0.00
XV	21-Acetoxy-20-ethylenedioxy-11β,16α,17α-trihydroxy-pregn-4-en-3-one	0.48	0.86
XVI	9α-Fluoro-11β,17α,21-trihydroxypregn-4-ene-3,20-dione	0.00	0.00
XVII	9α-Fluoro-11β,16α,17α,21-tetrahydroxypregn-4-ene-3,20-dione	1.00	1.00
XVIII	9α-Fluoro-11β,16α,17α,20β,21-pentahydroxypregn-4-en-3-one	0.79	1.00
XIX	16α,17α-Dihydroxy-17β-hydroxymethyl-D-homoandrost-4-ene-3,17a-dione	0.78[c]	
XX	16α,17aα-Dihydroxy-17aβ-hydroxymethyl-D-homoandrost-4-ene-3,17-dione	0.66[c]	
XXI	9α-Fluoro-11β,16α,17aα-trihydroxy-17aβ-hydroxymethyl-D-homoandrost-4-ene-3,17-dione	0.59[c]	0.74

[a] Anodic migration relative to that of 9α-fluoro-16α-hydroxycortisol (XVII).
[b] Slight migration towards the cathode.
[c] These migration rates were obtained at pH 8.0

which contains a 20β-hydroxyl group, migrated more slowly at lower pH values than the corresponding 20-ketosteroid (XVII).

Compound I, a 16α-hydroxy-17-ketosteroid, exhibited an anodic migration at alkaline pH, perhaps due to enolization of the ketone. It may be noted, however (Fig. 1), that at acid pH a slight cathodic migration occurred, similar to that observed with compounds II, III, VI and X. If enolization is a factor in the anodic migration of compound I, it is difficult to explain the lack of migration of 20,21-α-ketolic steroids, which might also be expected to enolize at alkaline pH.

C-20-Hydroxysteroids (cf. Table II)

Steroids containing a glycol- or glycerol-type side chain migrated toward the anode more slowly than 16α,17α-dihydroxysteroids. A 17α,20β-dihydroxysteroid (XXIII) travelled at twice the rate of a 20β,21-dihydroxysteroid (XXIV). The 17α,20α-dihydroxysteroid (XXII), epimeric at C-20 to compound XXIII, behaved erratically and streaked slightly toward the anode at pH 9.3. 17α,20β,21-Trihydroxysteroids (XXV, XXVII) migrated faster than the steroids containing the glycol-type side chains.

TABLE II

ANODIC MIGRATION OF C-20-HYDROXYSTEROIDS

Steroid		M_s[a]	
		pH 8.5	*pH 9.3*
XXII	17α,20α-Dihydroxypregn-4-en-3-one		slight streak
XXIII	17α,20β-Dihydroxypregn-4-en-3-one		0.21
XXIV	11β,20β,21-Trihydroxypregn-4-en-3-one		0.11
XXV	17α,20β,21-Trihydroxypregn-4-en-3-one	0.31	0.72
XXVI	20β,21-Diacetoxy-17α-hydroxypregn-4-en-3-one		0.00
XXVII	9α-Fluoro-11β,17α,20β,21-tetrahydroxypregn-4-en-3-one	0.23	0.69

[a] Anodic migration relative to that of 9α-fluoro-16α-hydroxycortisol.

Steroids containing 1,3- or 1,3,5-cis-oriented hydroxyl groups

Neither 14α,17α,21-trihydroxypregn-4-ene-3,20-dione nor 15α,17α,21-trihydroxypregn-4-ene-3,20-dione migrated in borate buffers. These steroids contain 1,3-*cis*-oriented hydroxyl groups, the former in the 14α- and 17α-positions and the latter in the 15α- and 17α-positions. However, 7α,14α,17α,21-tetrahydroxypregn-4-ene-3,20-dione, which has 1,3,5-*cis*-oriented hydroxyl groups at the 7α-, 14α- and 17α-positions, did migrate in pH 9.3 buffer with an M_s value of 0.44.

Analogous observations have been reported by ANGYAL AND MCHUGH[10], who found that 1,3,5-*cis*-trihydroxy cyclitols, but not 1,3-*cis*-dihydroxy cyclitols, moved to the anode during paper electrophoresis in borate buffer.

Electrophoretic migration in other electrolytes

Anodic migration of the 16α,17α-dihydroxysteroids was also observed in alkaline phosphate, barbiturate, and tris-(hydroxymethyl)-aminomethane buffers. The mobility of the steroids in each of these buffers was erratic and much lower than that in borate solution. No satisfactory explanation for this phenomenon can be given at this time.

SUMMARY

Certain polyhydroxylated steroids form negatively charged complexes in borate buffer which migrate toward the anode during paper electrophoresis. The relative migration rates are tabulated.

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MAGNIFICATION OF RESOLVING POWER OF COLLECTORS IN FREE FLOW ELECTROPHORESIS*

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The applicability of the device described in this paper is not limited to the serpentine flow bed method[1,2] and to the flow column stabilized by magnetic rotation[3,4] to which it has been applied so far. The following discussion will make it obvious how the proposed collection device can be used wherever the separated components of a mixture are distributed transversely to the fluid flow which carries them.

The problem to be solved is as follows: Let us assume that the flowing buffer carrying n separated components forms a thin fluid ribbon, say 2 mm in thickness and 5 cm in width. Let us suppose that the separated fractions form thin streaks in the buffer stream about $^1/_4$ mm in diameter. The buffer solution enters through the tube B (Fig. 1a) into the manifold M from where it is admitted to the separation chamber SC which it leaves through the collector tubes CT. IN is a hypodermic needle (gauge No. 22) through which the mixture to be fractionated is injected in a continuous stream which breaks up into the individual streaks S_1–S_4 carrying the components to be collected separately**.

It is obvious from inspection of Fig. 1a that one can get excellent resolution in the visible separation pattern without obtaining adequate resolution in the collection pattern. For example, if the streaks S_3 and S_4, each being $^1/_4$ mm in diameter, are separated by a gap of $^1/_4$ mm, they will be very clearly resolved in a photograph of the separation pattern, but they could easily pass out of the apparatus through one single exit tube CT of the collector if the latter were, for instance, 1 mm in internal diameter. Thus, there will be no separation of these components in the collection pattern.

In principle, resolution could be improved by making the adjacent collector tubes CT much narrower, spacing them as closely as possible, but this becomes practically very difficult. We have chosen, therefore, a different path to solve this problem. Instead of altering the lumen and the density of spacing of the collector tubes, the separation of the streaks was magnified by purely hydrodynamic means after completion of the electrophoretic separation. Fig. 1b shows the exit plate E of the buffer flow channel of Fig. 1a modified by omitting the exit tubes CT in the section Q_1. At this point, the flow channel is modified as shown in Figs. 1b and 1c.

* This work has been supported by a grant from the Office of Naval Research.
** This separation could, for instance, have been established electrophoretically or by any other method. For the sake of generality, the details of the separation apparatus are not indicated.

The buffer continues to flow between sections Q_1 and Q_2 through a trapezoidal channel at the end of which the exit tubes CT (of equal diameter and spacing as in Fig. 1a) are spaced at the same intervals as in Fig. 1a. This deformation of the flow channel leads to the deformation of the flow pattern indicated by the streaks S_1–S_4 in Fig. 1b. The intervals between the streaks are magnified approximately in the ratio of the cross-sections Q_2/Q_1 and the thickness of the streaks is similarly magnified. The latter magnification is no disadvantage since there is no objection to a separated fraction escaping through more than one collection tube. The collection of two ad-

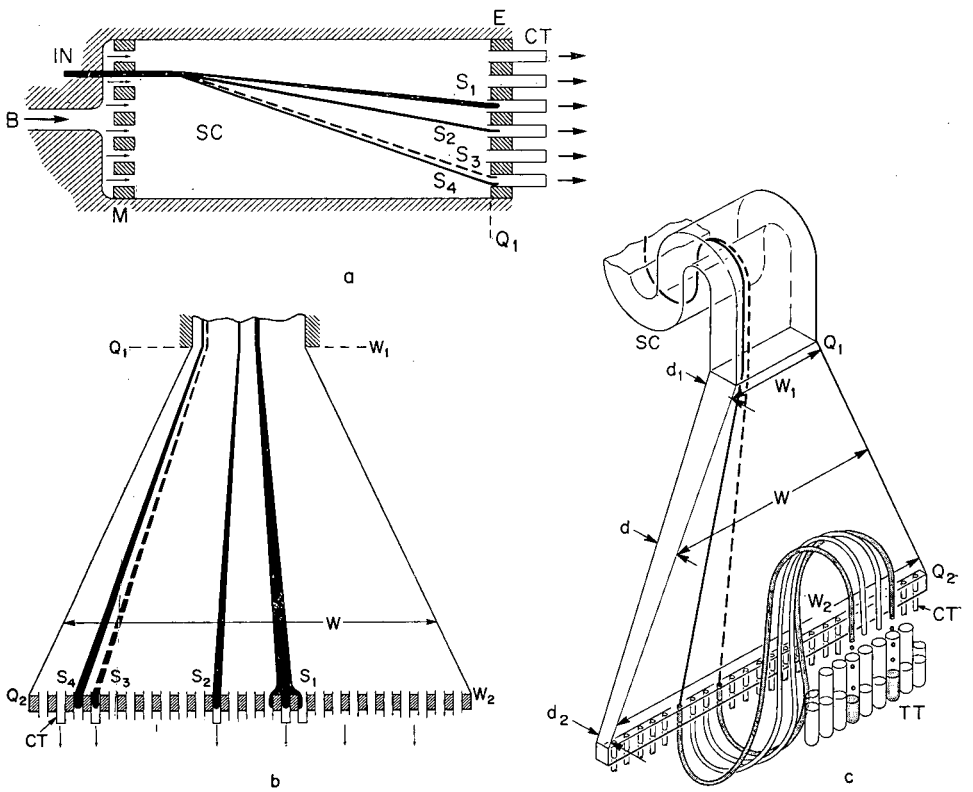


Fig. 1a. Continuous flow separation of four components S_1–S_4 entering from injector IN into the separation cell SC and leaving the cell through collector tubes CT. M = manifold. B = tube conveying the buffer solution. Q_1 = section at the location of the end plate E. The width at this point is 7 cm for the apparatus shown in Fig. 3.

Fig. 1b. The "fan" replacing the end plate E of Fig. 1a. Q_1 and Q_2 are the sections defining the origin and the end plate of the "fan", respectively. W_1 and W_2 are the widths of these sections. W = width of "fan" at an arbitrary point. S_1–S_4 = separated fractions. The separations between the fractions entering at section Q_1 are magnified purely by change of the flow pattern as they move toward section Q_2. At the same time, the streaks become wider. Components S_3 and S_4 which leave through the same collector tube in Fig. 1a, leave through separate collector tubes in Fig. 1b.

Fig. 1c. Perspective view of the "fan" with collector. SC = end section of the serpentine separation cell attached to the fan at section Q_1. The fan narrows down from depth d_1 at section Q_1 to depth d_2 at section Q_2. W and d = width and depth of the fan at an arbitrary location. CT = collector tubes. TT = test tubes of collector.

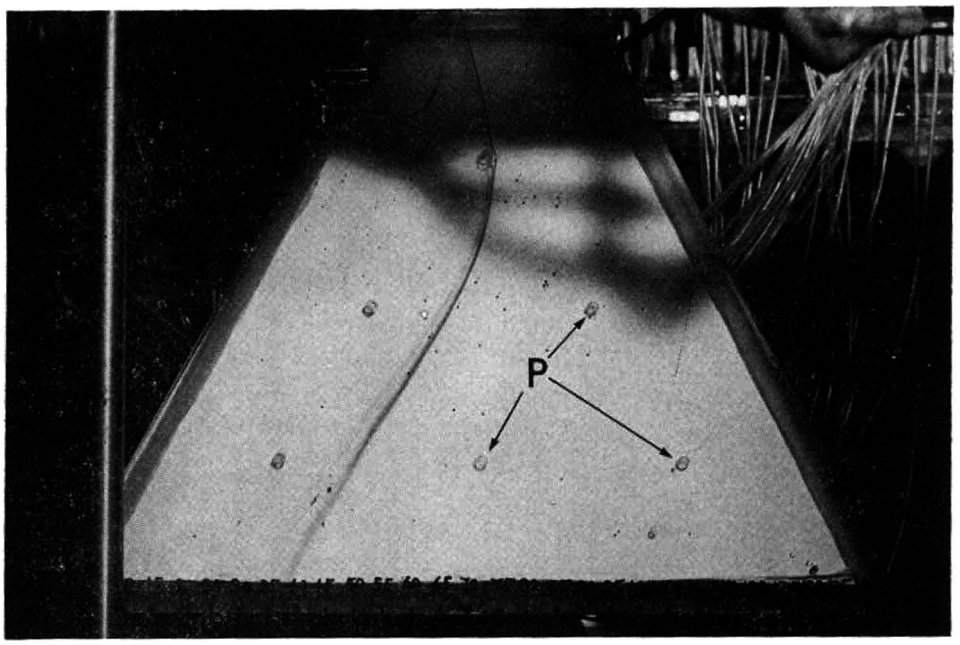
jacent fractions through one collection tube is, however, effectively avoided as shown by magnification of the gap between streaks S_3 and S_4 in Fig. 1b as compared to Fig. 1a. They are now far enough apart to be collected through different collector tubes.

To insure sufficient rigidity and parallelism between the trapezoidal plastic plates, small plastic pellets P, 2 mm in thickness, are cemented at several points between them as seen in Figs. 2a and 2b. These pellets do not disturb the separation process, they merely cause a deformation of streaks passing very close to them, which is of no consequence. This deformation is noticeable as a slight increase in curvature of the streaks seen in Figs. 2a and 2b at points of nearest approach to the pellets P.

We have to decide now what orientation of the trapezoidal channel will be best. A horizontal orientation is not recommended, especially with particulate components, because of gravitational sedimentation in the flow channel. Among the two possible vertical orientations, the one allowing the buffer to flow downward in the trapezoidal channel is preferable in electrophoretic separators since the buffer entering at Q_1 is still warm from exposure to the electrophoretic current; its temperature drops as it moves down in the trapezoidal channel and greater thermal stability is assured by an upward temperature gradient thus achieved. Nevertheless, if the current is large, thermal convection may still occur and may curve the streaks as shown in Fig. 2a. This can be avoided, as shown in Fig. 2b, and greater stability of the collection pattern can be achieved by submerging the trapezoidal "fan" into a cooling bath kept at a constant temperature (preferably 4°). An alternative method to cooling for suppression of thermal convection would be to use a serpentine "fan", *i.e.*, to use a serpentine flow bed as described in reference 1 allowing the width of the bed to increase in the downstream direction. Fig. 3 shows the apparatus without the cooling bath, which is merely a plastic box large enough to contain the "fan" and the collector tubes issuing from it.

The velocity of flow in the trapezoidal channel should be large as compared to velocities of possible thermal convection disturbances. The flow velocity decreases as we progress from the small cross-section Q_1 toward the wider and larger cross-section Q_2. One can achieve a uniform flow velocity in the trapezoidal channel by diminishing its depth as it becomes wider (*cf.* Fig. 1c). If we designate the depth of the channel by d, and its width in any cross-section by W, then the product $d \cdot W = d_1W_1 = d_2W_2 = C$ should remain constant to obtain the desired constancy of the flow velocity. It is not always practical to adjust the depth of the flow channel to safeguard constancy of the flow velocity. For instance, in the case of the apparatus shown in Figs. 1c and 3, assuming a ratio $W_2/W_1 = 4$ and $d_1 = 2$ mm, the channel exit should be reduced in depth to $^1/_2$ mm, which makes the construction of the device more difficult. Thus, as a compromise solution, the depth $d_2 = 1$ mm was chosen in the illustrated case with $d_1 = 2$ mm.

Figs. 1c and 3 show a trapezoidal collection "fan" used in conjunction with a serpentine separation cell[2]. Application to the magnetically stabilized rotating separation column will be described in a subsequent publication[4]. It is seen in Fig. 2b how the streaks of Evans blue (faster component on the left) and Rose Bengal, which are barely discernible at the entry into the "fan", are spread far enough apart at the exit to issue through non-adjacent collector tubes: No. 27 for Rose Bengal and No. 31 for Evans blue in a typical run (with slight amounts of dye entering the test tubes



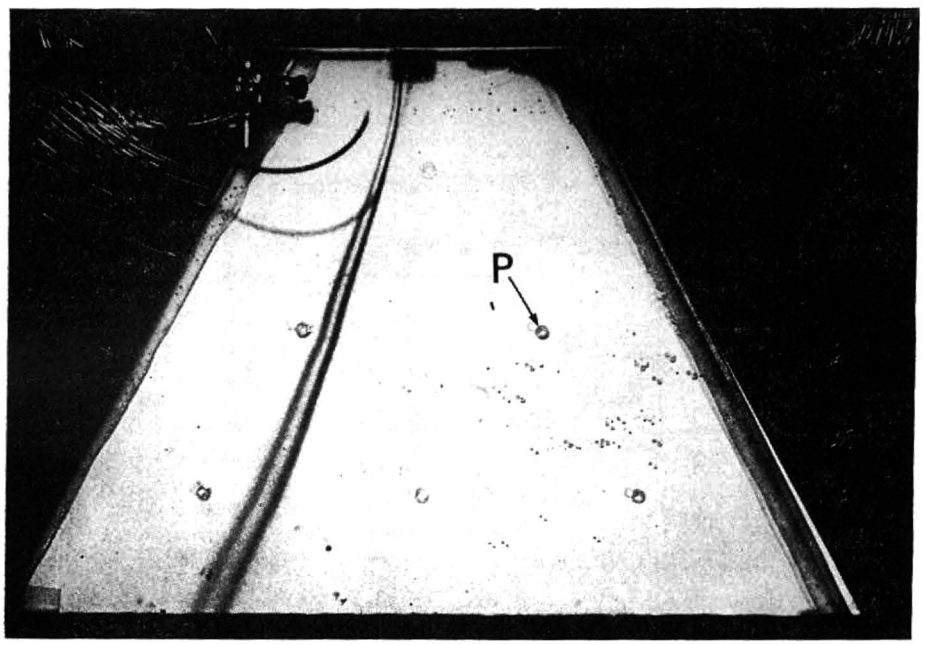


Fig. 2a. Separated fractions traversing the "fan" in the presence of thermal convection which causes the curvature of the streaks. P = lucite pellets safeguarding proper spacing between the walls of the "fan". The numbers at the bottom label the collector's exit tubes.

Fig. 2b. Inhibition of the thermal convection, shown in Fig. 2a, by immersion of the "fan" into a cooling bath (water temp. 16°). P = pellet (see Fig. 2a). The curvature of the streaks is slightly increased at the points of closest approach to a pellet. Left streak: Evans blue (faster component); right streak: Rose Bengal.

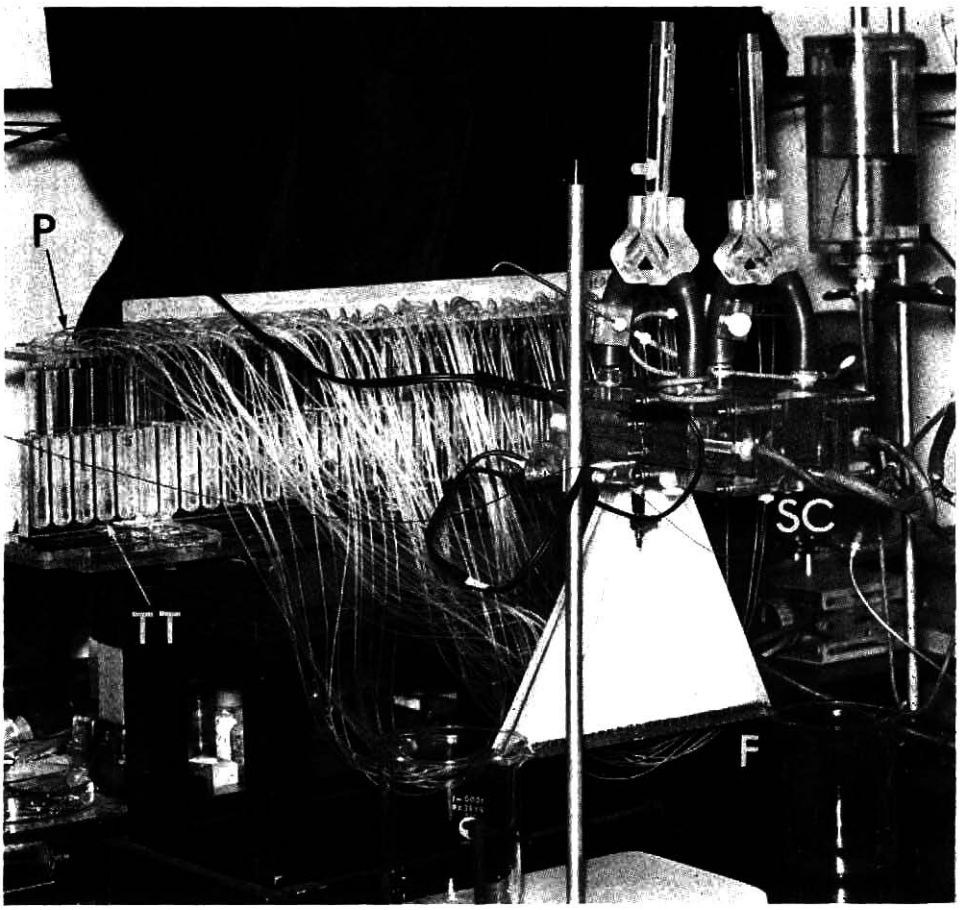


Fig. 3. View of a serpentine electrophoretic separator SC attached to the "fan" F. P = plate fixing the end points of plastic tubes collecting the outflow from the fan which is distributed among the test tubes TT. There are four such rows of tube endings. Only one row of test tubes is shown.

adjacent to No. 27 and No. 31). The total number of exit tubes is 143. They are linked by PVC tubing (approximately 1 mm I.D. and 2 mm O.D.) to 4 rows of holes in a plastic strip (P of Fig. 3) through which the tubes are passed so as to guide drops of issuing buffer into 4 rows of test tubes underneath. Only enough test tubes are used in practice to collect the desired components, the remaining buffer outflow is discarded. Only one row of test tubes is shown in Fig. 3.

Instead of inserting the exit tubes CT parallel to the flow into the terminal plate at Q_2 of Fig. 1c, they can be mounted at right angles to a wall of the "fan". This construction has been used in the device shown in Fig. 3. The tubes CT are mounted on the back side facing the test tube rack. This mode of construction is advantageous where the terminal interspace between the "fan" walls becomes too narrow as compared to the diameter of the tubes CT.

The general advantage of the device described above lies in the fact that one

can aim in practice at achieving only very narrow separation patterns by any type of method yielding continuous flow fractionation and spread this pattern rapidly by purely hydrodynamic means to dimensions permitting separate collection of the fractions.

I wish to acknowledge the technical assistance of Mr. PAUL COX in fabricating the "fan".

SUMMARY

By means of expansion of the width of the flow channel, it is possible to augment the resolving power of a collecting system in continuous flow fractionations. The device is applicable to any method of continuous flow fractionation based on injection of the mixture as a narrow streak. The operation of the method is demonstrated in the case of serpentine channel electrophoresis.

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THE PURIFICATION OF TESTICULAR HYALURONIDASE BY CHROMATOGRAPHY ON A MIXED COLUMN

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In a previous paper[1] the authors reported a method for obtaining a highly purified testicular hyaluronidase whereby in the last step of the purification procedure chromatography on diethylaminoethyl (DEAE)-Sephadex A-50 (medium) was used. As the method described involved quite a number of preliminary stages, we tried to avoid some of them by introducing a chromatographic procedure which could realize the desalting and the purification steps under simpler conditions and with greater efficacy. This was achieved by utilising a single column containing two layers, one consisting of the desalting gel and the other of the ion exchanger.

MATERIALS AND METHODS

The extract of the crude preparation of the hyaluronidase from the testicular material, the method of determination of the enzymic activity, and the preparation of the hyaluronic acid used as substrate were described in detail in our previous publication[1].

Preparation of the mixed chromatographic column

A 400 × 42 mm plastic column with a sintered filter was carefully packed up to a height of 150 mm with a DEAE-Sephadex A-50 (medium) suspension in phosphate buffer (pH 6.0, 0.02 M, $\mu = 0.08$)[1]. After the completion of the packing, in a vertical position, of the DEAE-Sephadex on to its upper, perfectly horizontal layer, a suspension of Sephadex G-75 (or G-100) in the same buffer is introduced, taking care not to disturb the surface. This gel layer is left to settle up to a height of 250 mm. The mixed column therefore consists of two layers, one above the other, with a total height of 400 mm, permitting a continuous flow during chromatography on two different gel suspensions. The upper layer (Sephadex G-75 or G-100) achieves the desalting stage by gel filtration[2], while the bottom layer carries out the purification by ion exchange (DEAE-Sephadex A-50*)[1].

RESULTS

The entire process of purification includes the following stages:

(a) Acetone-dried testicular material is passed through a meat grinder and extracted for 4 h at 4° with 0.1 N acetic acid–2 N hydrochloric acid (190:10) while stirring continuously.

* Supplied by Pharmacia, Uppsala, Sweden.

(b) The acid extract (protein: 10.6 mg/ml; activity: 2.40 turbidity reducing units (TRU) per mg protein) is centrifuged at 4° and the supernatant containing the crude enzyme is 30% saturated with ammonium sulphate and the precipitate discarded after centrifugation.

(c) The ammonium sulphate concentration in the supernatant is raised to 60% saturation and the mixture kept for 20 h at 4°.

(d) The precipitate obtained by 60% saturation is collected by centrifugation and, after solubilization in the same phosphate buffer as is used for the equilibration of the gels (pH 6.0, 0.02 M, $\mu = 0.08$), is submitted to the next step of purification.

(e) Chromatography on the mixed column (effected at 4°). The enzymically active protein solution (40 ml containing 13 mg protein/ml, with an activity of 57 TRU/mg protein and 7.6 g ammonium sulphate) was placed on the top of the column and introduced into it at intervals corresponding to a flow rate of 0.25 ml/min. Fractions of 5 ml were collected. The first 240 ml were free of protein and ammonium sulphate (tested by the Nessler reagent and also by barium chloride). The next 100 ml of effluent, collected at the same rate (0.25 ml/min), in 5 ml fractions, contained the enzymically active protein corresponding to 0.46 mg protein/ml and an activity of 12.751 TRU/mg protein, representing therefore a high degree of purity. The next 130 ml of effluent contained the largest amount of protein, but with very little enzyme activity (37 TRU/mg protein), the bulk of enzyme activity being confined to frac-

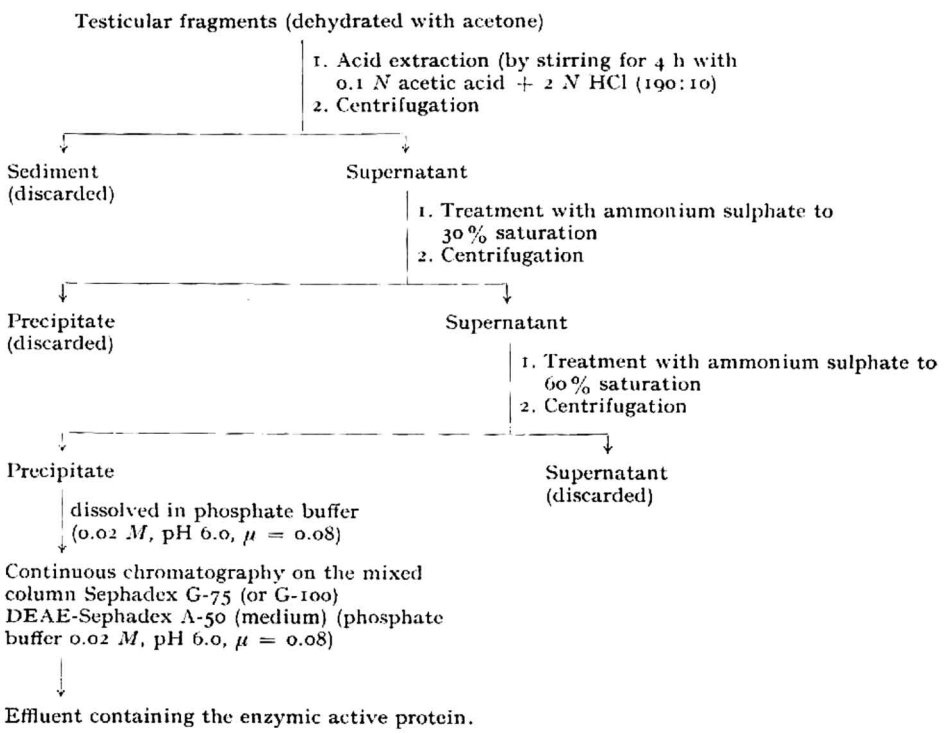

Fig. 1. Scheme of purification procedure for testicular hyaluronidase (all operations are carried out at 4°).

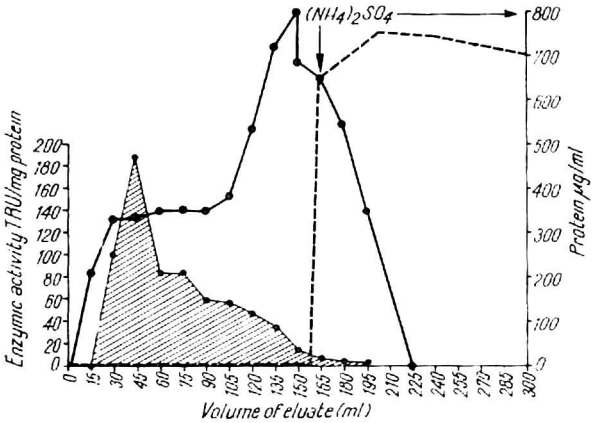

Fig. 2. Chromatography of testicular hyaluronidase on a Sephadex G-75 DEAE-Sephadex A-50 mixed column, 5 ml fractions collected (0.25 ml/min). Eluant: phosphate buffer (pH 6.0, 0.02 M, μ = 0.08). The hatched area corresponds to the fractions containing the active enzyme, the – ·—· –· area corresponds to the fractions containing the inactive protein, and the – – – area corresponds to the zone of the $(NH_4)_2SO_4$ effluent.

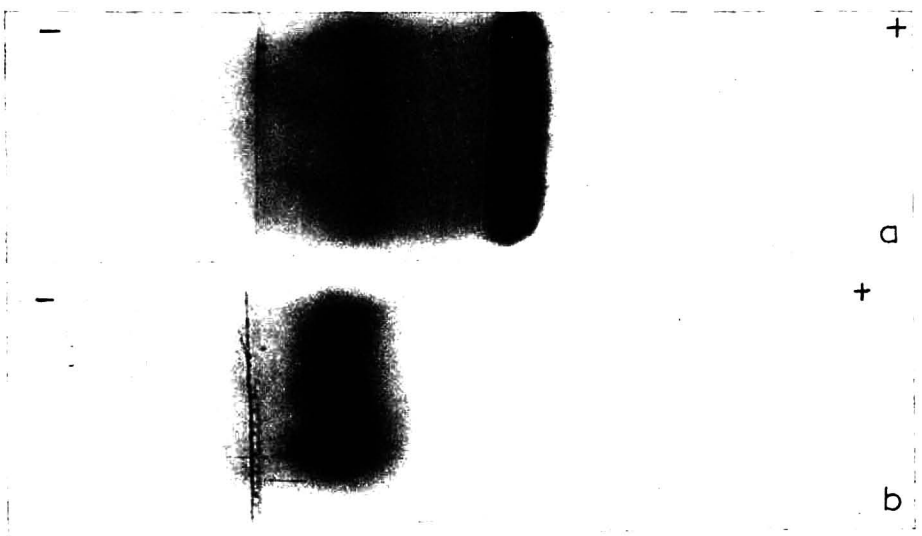

Fig. 3. Electrophoresis on Whatman No. 1 paper (0.06 M phosphate buffer, pH 6.0). (a) The crude preparation introduced into the column; (b) the eluates of the enzymically active fractions.

tions 1–20 (100 ml). The presence of ammonium sulphate could not be detected, either in the fractions containing the enzyme or in the fractions containing the inactive protein; it appears only in the subsequent fractions. The desalting process is thus efficient; the ammonium sulphate (7.6 g contained in the precipitate obtained at 60% saturation) remains absorbed on the column until the enzymically active proteins and the bulk of the inactive proteins have been completely eluted (reaction for ammonium ion with the Nessler reagent, for sulphate ions with the barium reagent). It was noted that the penetration of the ammonium sulphate into the DEAE-Sephadex layer has a shortening effect on it, but does not disturb the purification process. The purification stages described are summed up in Fig. 1. A representative experiment of a chromatographic separation on the mixed column where the desalting and purification of the crude testicular hyalyronidase is carried out in one step is illustrated in Fig. 2. The enzymically active protein is electrophoretically homogeneous (Fig. 3).

SUMMARY

A chromatographic method which achieves the desalting and purification of a crude hyaluronidase preparation (prepared by 60% ammonium sulphate saturation) in a single step by use of a mixed chromatographic column is described. The mixed column (400 × 42 mm) consists of a DEAE-Sephadex A-50 (medium) layer (150 mm in height), on top of which there is placed a Sephadex G-75 (or G-100) layer (250 mm in height); both layers are equilibrated with a phosphate buffer (pH 6.0, 0.02 M, $\mu = 0.08$). During the elution of the hyaluronidase with the same buffer, the inactive proteins and the ammonium sulphate were retained on the column; therefore an efficient purification of the enzyme can be achieved. The method described gives a good yield and a highly purified product under simplified experimental conditions.

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USE OF 2-ETHYLHEXYL PHENYLPHOSPHONIC ACID IN REVERSED PHASE PARTITION CHROMATOGRAPHY

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Two factors determine the separating ability of an extracting system in partition chromatography. The first is the effective height of the theoretical plate (later referred to simply as the height of the plate, H), the second is the separation factor for a pair of cations to be separated. It has been shown in previous papers from this Department[1,2] that the height of the plate depends on the flow rate, the temperature and the amount of extracting agent in the stationary phase. A comparison of our results, obtained for di-(2-ethylhexyl) orthophosphoric acid (HDEHP) as extracting agent, with the results of other authors working with the same extractant[3,4] shows also that the height of the plate depends on the nature of the supporting material and is lowest for silicone-treated kieselguhr. However, it is possible that this effect might not be caused by the different surface properties of the different materials, but simply by the different sizes of the grains of the supporting materials used by various authors. It also seems that the height of the plate may depend on the extracting agent itself because it is lower, on the average, for columns where tributyl phosphate (TBP) is used as extractant than for columns with HDEHP.

With respect to the separation factor for adjacent rare earths, HDEHP is vastly superior to TBP. However, PEPPARD, MASON AND HUCHER[5] have shown that 2-ethylhexyl phenylphosphonic acid (HEHØP) is an even more selective extracting agent for rare earths than HDEHP (the mean separation factor is 2.8, as compared with 2.5 for HDEHP). Provided that the height of the plate for columns with HEHØP supported on silicone-treated kieselguhr is not too great, HEHØP should be an excellent extracting agent for reversed phase partition chromatography of rare earths.

The possible applications of HEHØP in reversed phase partition chromatography of rare earths are discussed and the results are presented in this paper. A comparison with other systems has also been made.

EXPERIMENTAL

Silicone-treated kieselguhr "Hyflo Supercel" was used as the solid support for HEHØP. The column material was prepared as in the case of HDEHP[1] by mixing the appropriate amounts of kieselguhr and HEHØP with hexane, and evaporating the solvent at room temperature. The columns used in the course of this work were about 3 mm in diameter and the length of the bed was 9 cm. Such columns contained about 0.36 g of kieselguhr. The amount of HEHØP varied between 5 and 14 % relative to the weight of kieselguhr. The columns containing HEHØP as the extractant were perfectly stable and could be used for many runs.

The HEHØP used was kindly synthesised for us in the Department of Organic Chemistry at the Institute of Technology, Łódź. Its purity, as determined by titration, was 98 %. Most of the radio-isotopes used were prepared by irradiation of appropriate targets in the Polish reactor "EWA". Some radio-isotopes were obtained from the Radiochemical Centre, Amersham. The amount of the rare earth carriers was always below 0.001 mg. It was observed, in accordance with the results obtained for HDEHP, that the greater the amount of carrier the greater the influence on the shape and position of the elution peaks.

Most elutions were carried out at room temperature using acids pre-equilibrated with HEHØP. The flow rate was about 1 ml·cm^{-2}·min^{-1}. The volume of the sample introduced into the column was about 0.04 ml. The free volume of the column was determined using ^{137}Cs.

RESULTS AND DISCUSSION

The effect of various factors on the height of the plate

To determine the height of the plate the number of plates was calculated from the equation given by GLUECKAUF[6]

$$N = \frac{8\,V_{max}^2}{W^2}$$

where V_{max} is the volume of the eluate at the peak maximum and W is the width of the elution peak at 1/e of the maximum solute concentration.

No systematic study was performed on the effect of the grain size on the height of the plate. After some preliminary experiments a specific fraction of kieselguhr which settled within about 20 min in a 20 cm high beaker was selected. Using this fraction columns could be prepared which had a plate height of about 0.22 mm.

The height of the plate is affected by the amount of HEHØP retained on the kieselguhr in a similar way to that observed in the case of HDEHP[1]. This is illustrated by the data quoted in Table I. The height of the plate was calculated from the position and width of the europium peak. Elution was carried out with hydrochloric acid (0.95 M).

The influence of flow rate and temperature on the height of the plate is also shown in Table I. This seems to be even more pronounced than in the case of HDEHP.

Separation factors

Reversed phase partition chromatography allows the very exact determination of the separation factors from the positions of peak maxima. The results obtained for HEHØP are shown in Table II. The mean geometric separation factor calculated from these results is 2.50 for HCl as the eluting agent. Our data for HCl as the eluting agent show also that the mean separation factor for the rare earths from La to Gd is equal to 2.20 and is much lower than that for the rare earths from Gd to Lu, which in turn is equal to 2.85. The value of the mean separation factor for the rare earths from Gd to Lu is 3.02, when HNO_3 is used as the eluting acid.

The comparison of separation factors for the different groups of rare earths determined by the elution method is based on the assumption that separation factors

TABLE I

THE EFFECT OF FLOW RATE, TEMPERATURE AND AMOUNT OF HEHØP ON THE HEIGHT OF THE PLATE

Flow rate ($ml \cdot cm^{-2} \cdot min^{-1}$)	*Temperature* (°C)	*mg of HEHØP per 100 mg kieselguhr*	*Height of the plate* (*mm*)
0.25	20	14	0.11
0.50	20	14	0.16
1.0	20	14	0.22
1.0	20	14	0.22
1.0	25	14	0.19
1.0	30	14	0.16
1.0	20	9	0.20
1.0	20	14	0.22
1.0	20	18	0.25
1.0	20	30	0.45

are independent of the concentration of acid, because it is impossible to elute all the rare earths with one concentration of the acid. It has been verified in the course of this work that, at least up to 2 *M* HCl, the separation factors are independent of the concentration of acid, so that the values of separation factors for all the light rare earths are directly comparable. The same is true for the group of heavy rare earths since they were all eluted with HCl of constant concentration, using different amounts of HEHØP retained on the columns. But comparison of the mean separation factors for the two groups of rare earths includes some arbitrariness caused by the different concentrations of the eluting acid.

There are considerable deviations in the separation factors for different pairs of adjacent rare earths from the mean value. It is interesting to note that the sequence of positive and negative deviations from the mean value of the separation factor is very similar in the two groups of rare earths (La–Gd and Gd–Lu).

Examples of separations

In the course of this work, conditions for the separation of all rare earths in groups of several have been established. Fig. 1–4 show examples of such separations including all those pairs of the adjacent rare earths which have low values for the separation factors. As follows from Table II there are six such pairs: Ce–Pr, Pr–Nd, Eu–Gd, Tb–Dy, Dy–Ho and Yb–Lu. In spite of the low values of the separation factors all these separations are excellent. In the case of the heavy rare earths a smaller amount of HEHØP was used (9 % and 4.7 % relative to the amount of kieselguhr) to decrease the concentration of acid necessary for elution.

Comparison with other systems

Rare earths can be separated by the method of reversed phase partition chromatography using TBP, HDEHP and HEHØP as extractants. From the point of view of separation factors the system in which HEHØP is used as the extractant is the best, although the difference between HEHØP and HDEHP under the conditions

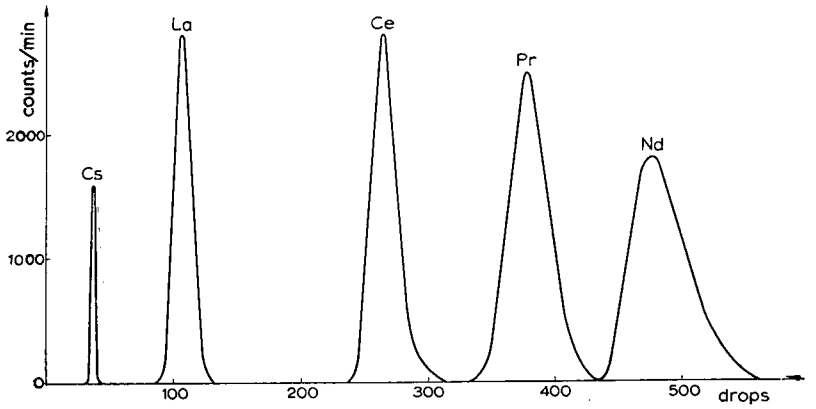

Fig. 1. Separation of La–Ce–Pr–Nd with 0.42 *M* HCl at 20°. Amount of HEHØP: 14% (relative to the weight of kieselguhr).

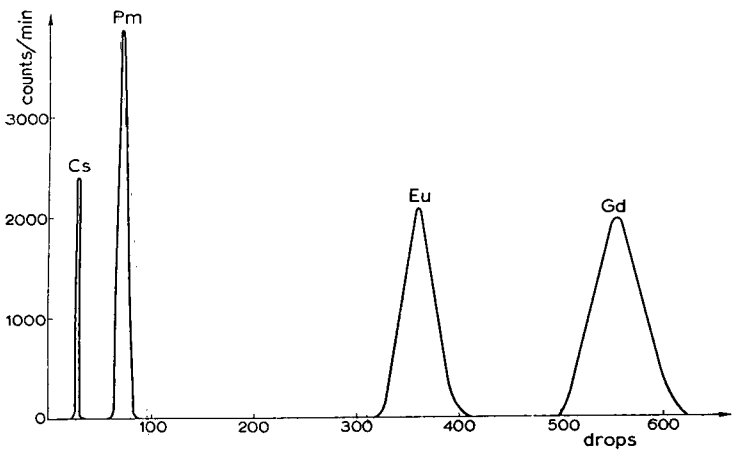

Fig. 2. Separation of Pm–Eu–Gd with 0.95 *M* HCl at 29°. Amount of HEHØP: 14%.

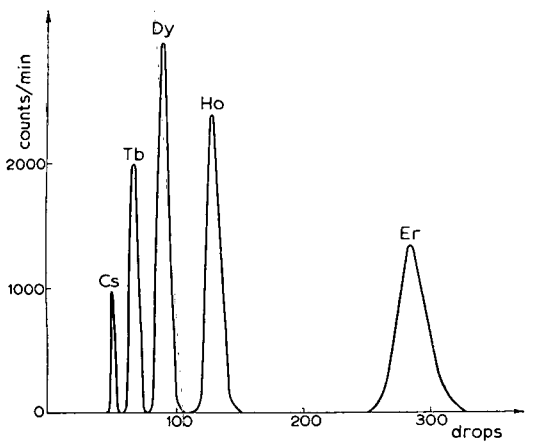

Fig. 3. Separation of Tb–Dy–Ho–Er with 7.4 *M* HNO_3 at 20°. Amount of HEHØP: 9%.

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TABLE II

SEPARATION FACTORS FOR ADJACENT RARE EARTHS

	La–Ce	*Ce–Pr*	*Pr–Nd*	*Nd–Pm*	*Pm–Sm*	*Sm–Eu*	*Eu–Gd*	*Gd–Tb*	*Tb–Dy*	*Dy–Ho*	*Ho–Er*	*Er–Tm*	*Tm–Yb*	*Yb–Lu*
Elution with HCl														
Separation factor	3.3	1.5	1.3	2.8	3.6	2.3	1.6	5.4	2.1	1.9	2.9	3.8	3.2	3.0
Molarity of acid	0.42	0.42	0.42	0.66	0.66	0.95	0.95	1.94	6.58	6.58	6.58	6.58	6.58	6.58
	0.57	0.57	0.57	0.95	0.95		1.94		7.06	7.06	7.06	7.06	7.06	7.06
									7.60	7.60	7.60	7.60	7.60	7.60
Elution with HNO_3														
Separation factor							1.8	5.9	2.3	2.0	3.0	4.0	3.4	2.1
Molarity of acid							2.06	2.06	7.39	7.39	7.39	7.39	7.39	7.39

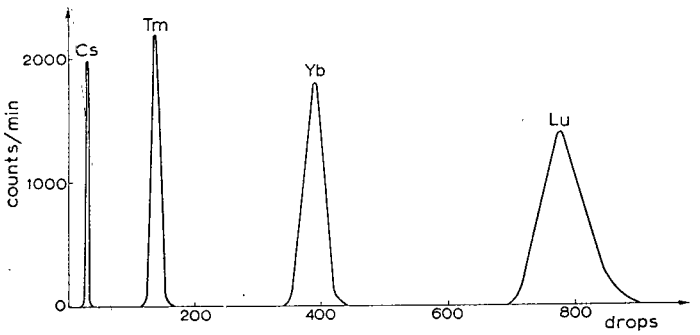

Fig. 4. Separation of Tm–Yb–Lu with 7.4 *M* HNO_3 at 20°. Amount of HEHØP: 4.7%.

used is rather small. Both systems show higher separation factors for the rare earths from Gd to Lu than from La to Gd (the values for HDEHP are: 2.05 and 2.81[1]; elution carried out with HCl). Because of the separation factor, HNO_3 is a better eluting agent for the group of heavy rare earths than HCl. In the system with HDEHP as the extractant, HCl cannot be used at all for the elution of the heavy rare earths, because of the broadening of the elution peaks. This effect was not observed in the case of HEHØP as the extractant. HCl, in turn, is a better eluting agent for light rare earths in the HDEHP system. HEHØP is a stronger extracting agent than HDEHP, so that more concentrated acid must be used for the elution, or a smaller amount of HEHØP must be placed on the column. In the latter case the capacity of the column is decreased. As far as the separation factors are concerned the system with TBP as extractant is less favourable for the separation of heavy rare earths than the two systems with acidic extractants. The mean separation factor in the system TBP–HNO_3 for the rare earths from Gd to Lu is 1.5 and that for light rare earths is about 1.9. It should also be noted that for the separation of the light rare earths in the system TBP–HNO_3, highly concentrated acid must be used as an eluant, which is very inconvenient in some applications. Besides, at such high concentrations of HNO_3 columns are unstable and can only be used for few experiments.

As regards the second factor determining the separating ability of a system, TBP seems to be somewhat more favourable than the two acidic extractants. The height of the plate for columns with TBP as the stationary phase can be as low as 0.15–0.14 mm, whereas for columns containing HDEHP or HEHØP it is generally not less than 0.22 mm. Since for columns containing tri-*n*-octylphosphine oxide (TOPO) the height of the plate is also very low, it seems that neutral extracting agents are better in this respect than acidic extractants. The system with TBP also seems to be less sensitive to the amount of carrier.

Nevertheless the higher separation factors and the lower concentrations of acid needed for elution together with a sufficiently low value of the plate height make the acidic extractants, and especially HEHØP, the best extractants for the separation of trace amounts of rare earths by reversed phase partition chromatography.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. Dr. J. MICHALSKI from the Department of

Organic Chemistry at the Institute of Technology, Łódź for kindly supplying the HEHØP. Thanks are also given to Mrs. R. OSIŃSKA for her skilled assistance.

SUMMARY

Reversed phase partition chromatography with 2-ethylhexyl phenylphosphonic acid retained on kieselguhr as the stationary phase was applied to the separation of rare earths. A comparison with other extracting agents used in partition chromatography has been made.

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ADSORPTION ON INORGANIC MATERIALS

VI. REACTION OF INSOLUBLE SULFIDES WITH METAL IONS IN AQUEOUS MEDIA*

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The favorable properties of precipitated CdS (when used without diluents) for the recovery of several metal ions which form less soluble sulfides were discussed in an earlier communication[1]. At room temperature (25°) the exchange rates were found to be fast, and quantitative removal of a number of heavy metal ions was achieved from dilute and concentrated solutions essentially through metathetical reactions.

The present paper describes the reaction of precipitated sulfides of Ag(I), Fe(II), Cu(II), Zn(II), Pb(II), Cd(II), and As(III) with solutions containing macro amounts of heavy metal ions, such as Ag(I), Cu(II), and Hg(II), which form highly insoluble sulfides**. The ease and extent of substitution of metal ions by many of the sulfides seems noteworthy.

Although displacement reactions of this type have been studied for sulfide minerals, such as sphalerite[3–8] and galena[9] it was generally believed that these were surface reactions and, hence, that they occur only to a small extent. To improve the extent of reaction while maintaining the speed of surface reactions for chromatographic purposes, some workers have employed devices such as impregnation of papers with CdS[10,11], cellulose (cotton) with CdS[12–15] or ZnS[16] and Filter Cel with ZnS[17]. High-fired ZnS as a "filter-medium" was studied by KUTZELNIGG[18]. He examined reactions with a number of metal ions in solution. While he did not report flow rates used or uptakes, we conclude from data presented in his tables that he was dealing with low degrees of conversion (usually less than 0.1 moles/l of bed). RYABCHIKOV and co-workers[19] reported as part of a routine study on selective "sorption" by a number of inorganic materials that CdS selectively adsorbs Cu(II) ions. This was thought to be a unique reaction.

In the present paper we would like to show that these reactions are not unique and that they frequently occur with high degrees of conversion even when the materials are not specially treated, or dispersed as thin layers on supports or diluents.

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* Research jointly sponsored by the Office of Saline Water, U.S. Department of the Interior, and U.S. Atomic Energy Commission under contract with the Union Carbide Corporation. For the previous paper, see ref. 1.

** According to W. M. LATIMER[2], the solubility products of the sulfides are: Hg(II) $1.6 \cdot 10^{-54}$; Ag(I) $5.5 \cdot 10^{-51}$; Cu(II) $8 \cdot 10^{-37}$; Pb(II) $7 \cdot 10^{-29}$; Cd(II) $1.0 \cdot 10^{-28}$; Zn(II) $7 \cdot 10^{-26}$ (sphalerite); FeS $4 \cdot 10^{-19}$.

EXPERIMENTAL

Most sulfides were prepared by addition, with stirring, of excess ammonium sulfide to 0.2 to 0.4 *M* solutions of the metal ions; As(III) sulfide was prepared by the addition, with stirring, of excess H_2S gas to a 0.2 *M* As(III)–0.7 *M* HCl solution. The resultant precipitates were filtered, washed with water, dried at 25°, ground and sieved (80–170 mesh). Small columns (*ca.* 0.2 cm^2 × 1 to 2 cm) made from these materials (without use of diluents) have reasonably good flow characteristics. Some required a few lb/in.^{2} over-pressure for satisfactory flow. Bed densities (g/cc) were: AgS: 1.5; FeS: 1.0; CuS: 1.5; ZnS: 1.4; CdS: 0.9; PbS: 1.6; As_2S_3: 0.5.

The effluents were usually analyzed for displaced metal ions by EDTA titrations; Ag(I) was determined by Volhard titration (SCN^-), Fe(II) by titration with $KMnO_4$, and As(III) by iodimetric titration in neutral solutions. Radiometric analyses were used to determine the breakthrough of added metal ions. Radiotracers (^{64}Cu, ^{110}Ag, ^{203}Hg) were obtained from the Isotopes Division of Oak Ridge National Laboratory and used without further purification though checked for purity. The stoichiometry of the reactions was obtained from the effluent analyses and the weight of sulfides used.

Composition of the solid was taken as the stoichiometric sulfide after drying at 105° for 24 h. The weight loss on drying was 6.7 % for ZnS and about 1 % for the other sulfides.

RESULTS AND DISCUSSION

We shall first discuss the stoichiometry of the reactions of various solid sulfides with solutions of adsorbable ions. We shall then present results concerning the uptakes of ions by the solids on a weight and volume basis; some comments on the rates of reactions are also included. Finally, we show that the adsorption properties of sulfides are not very well correlated with their structure and crystallite size.

(1) *Stoichiometry of displacement reactions*

Small columns of the sulfides were found to remove quantitatively, from a variety of solutions, metal ions [*e.g.*, Ag(I), Cu(II), Hg(II), Au(III)] which form more insoluble sulfides. The stoichiometry of the reactions can frequently be approximated by the metathetical reaction:

$$M_{2/m}S + 2/n\ N^{+n} \longrightarrow 2/m\ M^{+m} + N_{2/n}S \qquad (1)$$

where M^{+m} is the metal ion in the original sulfide and N^{+n} is the displacing ion.

The exchange equivalence of the reactions was studied for a number of systems by determining the conversion ratio *R* of the number of moles of metal ions displaced from the solids to the number of moles of metal ions retained or "adsorbed".

(*a*) *Ag_2S.* Silver sulfide was found to react sufficiently rapidly with added Hg(II) to cause essentially quantitative removal of this element from solution. A typical adsorption–displacement experiment is illustrated in Fig. 1 which gives the conversion ratio *R* of moles Ag(I) eluted per mole Hg(II) adsorbed from a 0.053 *M* $Hg(NO_3)_2$–0.0002 *M* HNO_3 solution. Flow rate was 1 cm/min; temperature 25°.

According to eqn. (1), elution of 2 moles of Ag(I) per mole of Hg(II) would have been expected. Since $R < 2$, there is either some adsorption of excess Hg(II) on the HgS formed or some adsorption of Ag(I) on HgS; both reactions seem possible.

(*b*) *CuS*. Adsorption experiments were carried out with solutions of Ag(I) and Hg(II). A typical experiment with 0.098 *M* $AgNO_3$–0.1 *M* HNO_3 is illustrated in Fig. 1. Flow rate was 0.3 cm/min, temperature 25°. Copper(II) was found immediately in the effluent and R was 0.5 as expected from eqn. (1).

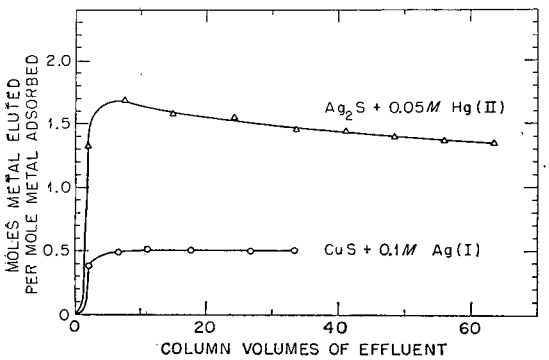

Fig. 1. Reaction of Hg(II) with Ag_2S and Ag(I) with CuS. Column 0.2 cm^2 × 1.2 cm; flow rate 0.3–1 cm/min; 25°.

Adsorption of Hg(II) by CuS was relatively slow at room temperature. At 97° essentially quantitative adsorption occurred for many column volumes with 0.05 *M* Hg(II)–0.0002 *M* HNO_3 solution at a flow rate of 0.9 cm/min. The conversion ratio ($R \approx 0.5$) was substantially less than expected from eqn. (1) and dependent on flow rate. Apparently the HgS forming during the reaction adsorbed excess Hg(II). This at least in part is due to formation of double salts of the type $2Hg_2S \cdot HgX_2$ (where X is an anion such as nitrate) since these double salts are white[20] and the columns slowly changed in color from black to grey.

(*c*) *ZnS*. Silver ions are quantitatively retained (or adsorbed) by a ZnS column; a black band is formed which, at reasonable flow rates, has a sharp frontal edge. Although some Zn(II) appeared immediately in the effluent, the conversion ratio R only gradually approached the theoretical value of 0.5 predicted from eqn. (1). A typical experiment with a 0.048 *M* $AgNO_3$–1 *M* $NaNO_3$–0.001 *M* HNO_3 solution is illustrated in Fig. 2. Temperature was 25° and flow rate 5 cm/min. There is apparently some retention of displaced Zn(II), perhaps through reaction with some excess sulfide incorporated in the precipitate.

Uptake of such "excess" Zn(II) by the material was demonstrated by passing a 0.044 *M* $Zn(NO_3)_2$ solution through a similar ZnS column. Fifty percent breakthrough of Zn(II) occurred after passage of 13 column volumes (c.v.) which corresponds to an adsorptive capacity of the solid of 0.4 moles Zn(II) per kilogram. While this value seems large, it must be recognized that it represents only 4% of the theoretical conversion capacity of the solid. If this adsorption of excess Zn(II) is indeed due to excess sulfide in the solid, this adsorption technique would constitute an exceedingly sensitive method of analysis of such solids.

On addition of silver nitrate solution [0.048 M Ag(I)–1 M $NaNO_3$–0.001 M HNO_3] to such a Zn(II) pretreated ZnS, the conversion ratio R reached 90 % of the theoretical value [eqn. (1)] within 1 c.v. — *i.e.*, much more rapidly than with the untreated material. However, a small discrepancy between observed and predicted conversion ratios remained, implying a residual reaction of Zn(II) with the silver sulfide formed or some other parasitic reaction.

Removal of Cu(II) from solution by adsorption on ZnS was tested with nitrate

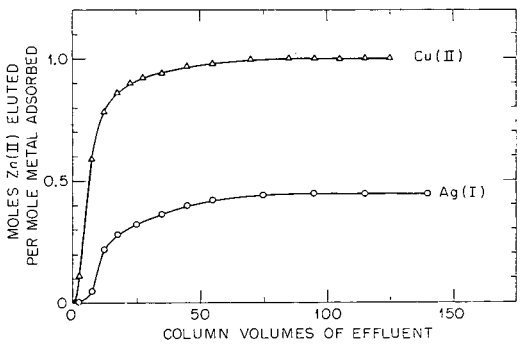

Fig. 2. Reaction of Ag(I) and Cu(II) with ZnS. Column 0.2 cm^2 × 1.0 cm; 0.05 M metal solutions; flow rate *ca.* 5 cm/min; 25°.

solutions. A typical experiment involving addition of 0.052 M $Cu(NO_3)_2$–0.004 M HNO_3 is also illustrated in Fig. 2. Flow rate was *ca.* 5 cm/min, temperature 25°. On addition of the Cu(II) solution rapid conversion occurred with the formation of a black band with a sharp frontal edge. Although some Zn(II) appeared immediately in the effluent, the conversion ratio, as in the case of Ag(I), only slowly approached the theoretical value ($R = 1$).

Adsorption of Hg(II) by ZnS seems more complicated. Tests were made with *ca.* 0.053 M $Hg(NO_3)_2$–0.002 M HNO_3 solutions at 25°. Under these conditions, Hg(II) breakthrough occurred after 10 c.v. at a flow rate of 0.3 cm/min. While Hg(II) was absorbing, the column underwent a series of color changes (orange to black to grey) implying that a variety of compounds is formed. At elevated temperatures (97°) these color changes were faster but Hg(II) still showed significant breakthrough after approximately 10 c.v. Effluent analyses at both temperatures yielded very low values of R (*ca.* 0.1), presumably because of a combination of Zn(II) adsorption on ZnS and Hg(II) adsorption on the HgS produced.

(*d*) *CdS.* The stoichiometry for the displacement of Cd(II) from its sulfide by Ag(I), Cd(II) and Hg(II) was described earlier[1].

In more recent experiments, Au(III) (0.022 M $AuCl_3$–0.6 M HCl) was also found to react quantitatively with CdS to give a black band with sharp frontal edge. Displaced Cd(II) appeared immediately in the effluent (Fig. 3). The value for the conversion ratio, R, increased to about 1.1 after 19 c.v. Addition of more Au(III) was accompanied by a gradual decrease in R until after *ca.* 60 c.v. it approached an asymptotic value of 0.80. Presumably some of the Au(III) was retained by side reactions.

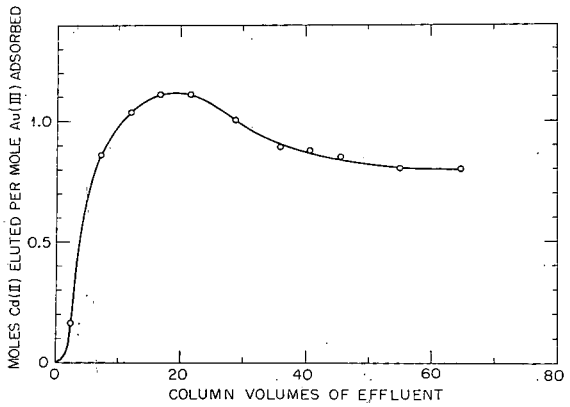

Fig. 3. Reaction of Au(III) with CdS. Column 0.2 cm^2 × 1.0 cm; 0.02 *M* Au(III); flow rate 3 cm/min; 25°.

(*e*) *PbS*. Reaction of PbS with Ag(I), Cu(II) and Hg(II) was found to be rapid and quantitative with small columns. The conversion ratios for Ag(I) and Cu(II) rapidly approached the theoretical values of 0.5 and 1.0 respectively as illustrated in Fig. 4 for 0.048 *M* $AgNO_3$–1 *M* $NaNO_3$–0.001 *M* HNO_3 and 0.052 *M* $Cu(NO_3)_2$–0.004 *M* HNO_3 solutions. The conversion ratio *R* in the case of Hg(II) [0.053 *M* $Hg(NO_3)_2$–0.0002 *M* HNO_3] was close to unity for the first 30 c.v. and then decreased to *ca.* 0.65. The color of the column slowly turned grey, presumably because of formation of the double salt $Hg(NO_3)_2 \cdot 2\,HgS$.

(*f*) *FeS*. Iron sulfide reacted sufficiently rapidly with Ag(I) (0.050 *M* $AgNO_3$), Cu(II) [0.052 *M* $Cu(NO_3)_2$–0.004 *M* HNO_3], and Hg(II) [0.053 *M* $Hg(NO_3)_2$–0.002 *M* HNO_3] to remove them quantitatively from their solutions at flow rates from 1 to 3 cm/min. Fe(II) appeared in the effluent in the expected ratio except in adsorption experiments with Hg(II) where *R* was about 90 % of theoretical.

(*g*) As_2S_3. As shown in Fig. 5, addition of Ag(I) [0.048 *M* $Ag(NO_3)_2$–1 *M* $NaNO_3$–0.001 *M* HNO_3] to As_2S_3 gave quantitative adsorption at a flow rate of 7 cm/min; a black band with sharp frontal edge formed. After a few c.v. the conversion ratio reached the asymptotic value 0.33 as expected from eqn. (1). While

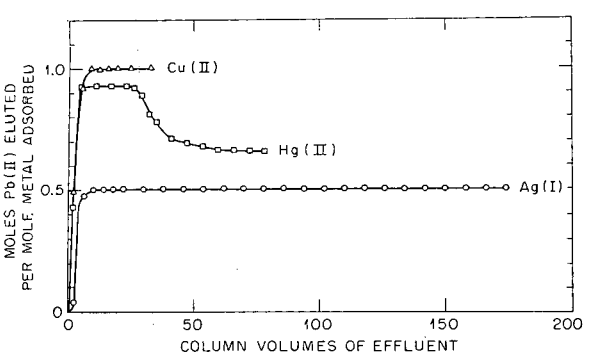

Fig. 4. Reaction of Ag(I), Cu(II) and Hg(II) with PbS. Column 0.2 cm^2 × *ca.* 1.4 cm; 0.05 *M* metal solutions; flow rate *ca.* 5 cm/min; 25°.

addition of Cu(II) [0.052 *M* $Cu(NO_3)_2$–0.004 *M* HNO_3] yielded a well defined dark band with sharp frontal edge at a flow rate of 3 cm/min, the extent of reaction was small and copper breakthrough occurred after *ca.* 7 c.v. As(III) appeared immediately in the effluent and reached an apparently asymptotic value $R = 0.75$ after 3 c.v. It is not clear why this value is larger than the theoretical value 0.67 expected from eqn. (1).

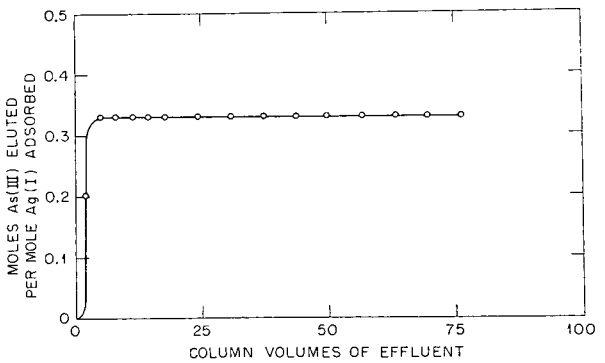

Fig. 5. Reaction of Ag(I) with As_2S_3. Column 0.2 cm^2 × 1.5 cm; 0.05 *M* Ag(I); flow rate 7 cm/min; 25°.

With Hg(II) [0.053 *M* $Hg(NO_3)_2$–0.0002 *M* HNO_3] removal was quantitative only for *ca.* 13 c.v. at a flow rate of 4 cm/min. There was no significant color change at first. Only after *ca.* 4 months did the column turn black. The conversion ratio *R* was *ca.* 0.3— *i.e.*, less than half the value predicted from eqn. (1).

(2) *Uptakes and rates*

The moles of metal per kg solid "adsorbed" by small (1–2 cm) columns of Ag(I), Cu(II), Zn(II), Pb(II), Cd(II) and As(III) sulfides varied from 0.5 to 14.5 at 50 % breakthrough (Table I) for the solutions given in Section 1 and at the flow rates given.

Values for the uptake by iron sulfide were variable and hence are not given in Table I. This variability may have been caused by partial oxidation of the original sulfide (FeS?) to ferric oxide and sulfur during the air-drying step. Some average values for metal ion uptakes (at 50 % breakthrough) by "iron sulfide" are Ag(I):5.8 moles/kg; Cu(II):2.0 moles/kg; Hg(II):2.5 moles/kg.

Most uptakes by the sulfides are enormous compared with standard ion exchange resin capacities. Particularly striking is uptake achieved with Ag(I); for the sulfides tested, it ranged between 5 and 20 moles per l of bed at 50 % breakthrough.

Indeed, for the reaction of Ag(I) with columns of many of the sulfides, flow rates as high as 50 cm/min were possible without smearing the frontal edge of the adsorption bands. Very high flow rates were also feasible for Hg(II) with PbS and Cu(II) with CdS and PbS. Except for CuS and As_2S_3 the uptakes of Ag(I) correspond to substantially more than 50 % of the value expected for the displacement reaction eqn. (1). For As_2S_3, the lower value obtained might imply that the final compound formed was Ag_3AsS_3 rather than Ag_2S which seems to be the case with the sulfides of Zn, Cd and Pb.

TABLE I

UPTAKE OF A NUMBER OF METALS BY VARIOUS SULFIDES (25°)

Sulfide	*Metal ion*	*Flow rate (cm/min)*	*Uptake at 50 % breakthrough*			
			Moles/kg	*Moles per l/bed*	*Moles per mole M_xS_y*	*% of theoretical [eqn. (1)]*
Zn	Ag(I)	7	14.5	20.3	1.4	71
	Cu(II)	4	6.6	9.3	0.6	65
	Hg(II)	0.3	0.5	0.8	0.05	5
Cd	Ag(I)	3	13.5	12.1	1.95	98
	Cu(II)	5	4.8	4.3	0.7	69
	Hg(II)	2	4.4	4.0	0.6	64
	Au(III)	3	3.6	3.2	0.5	78
Pb	Ag(I)	5	7.1	11.4	1.7	85
	Cu(II)	5	1.8	2.9	0.4	43
	Hg(II)	5	4.2	6.7	1.0	98
As	Ag(I)	7	11.1	5.5	2.7	46
	Cu(II)	3	0.9	0.5	0.2	7
	Hg(II)	4	2.1	1.1	0.5	17
Cu	Ag(I)	0.3	3.2	4.9	0.3	15
	Hg(II)*	0.9	3.3	5.0	0.3	32
Ag	Hg(II)	1	5.1	7.8	1.3	128

* Adsorbed at 97°.

Copper uptake by the various sulfides was consistently less than silver uptake. In the case of its reaction with ZnS, CdS and PbS very high uptakes were, however, achieved. With As_2S_3 the extent of reaction was so small as to imply that the reaction of Cu(II) occurs here only on the surface of the particles and that reaction within the particles is effectively arrested.

Uptake of Hg(II) by various sulfides was complicated because of the possible reaction of the HgS formed with additional Hg(II) to form double salts which could lead to "capacities" in excess of unity if computed with eqn. (1). In spite of this possibility, ZnS, As_2S_3 and CuS showed surprisingly low uptake of Hg(II)* at room temperature. This is particularly striking in the Hg(II)–ZnS system, since for it the free energy of the metathetical reaction is by far the largest but extent of reaction particularly small. Apparently reaction of Hg(II) with these sulfides occurs principally on the surface of the particles.

Uptake of Au(III) was tested only with CdS. At 50 % breakthrough uptake corresponded to 78 % of theoretical. In some preliminary experiments good adsorption was also found for platinum and bismuth with CdS.

(3) Crystal size and structure

Crystallite sizes were determined** for solids as precipitated and dried at 25° and for some solids after conversion (Table II). The observed rates of adsorption do not correlate in any direct and obvious way with the crystallite sizes or structure of

* In the reaction of Hg(II) with As_2S_3 the solid remained yellow at first which implies that the surface compound formed initially could be $Hg(AsS_2)_2$. This compound is yellow and has been described as a product of the reaction of Hg(II) with arsenic sulfide. See ref. 21.

** Crystallographic information was provided by R. L. SHERMAN of the ORNL Analytical Chemistry Division using X-ray low-angle scattering technique.

the sulfides. Although the ZnS crystallite size was smaller than that of CdS, the rates of conversion with Ag(I), Cu(II) and Hg(II) were all more rapid with CdS. Similarly, since ZnS and CdS may belong to the same zinc-blende type of structure, Hg(II) might be expected to react with them in about the same manner. However, as shown in Table I, Hg(II) converted ZnS about 5 % at 50 % breakthrough, while it converted CdS about 64 % at the same percent breakthrough and at a flow rate nearly 20 times faster.

TABLE II

STRUCTURE AND CRYSTALLITE SIZE FOR SULFIDES BEFORE AND AFTER CONVERSION

Sulfide	*Structure (initial)*	*Size (Å) (initial)*	*Converting ion*	*Structure (final)*	*Crystallite size (Å) (final)*
Zn(II)	Zinc-blende	~ 30	Ag(I)	Monoclinic	~ 430
Cd(II)	Zinc-blende	~ 70	Ag(I)	Monoclinic	550
			Cu(II)	Hexagonal	230
Pb(II)	Sodium chloride	215	Ag(I)	Monoclinic	~ 470
As(III)	Amorphous	20	Ag(I)	Monoclinic	~ 360
Cu(II)	—	~ 600	Ag(I)	Monoclinic	~ 700
			Hg(II)	—	~ 250
Ag(I)	Monoclinic	~ 290	Hg(II)	—	~ 250

The data in Table II show that the crystallite sizes of many converted sulfides were much larger than those of the original sulfides and, hence, that there was substantial crystal growth during the metathetical reaction. This would imply that the initial materials though largely amorphous, perhaps with crystalline "centers", have amorphous regions which are relatively continuous over many hundreds of Å and that during the reaction a large portion of this larger amorphous particle is transformed to a crystalline particle.

The rate and extent of conversion of these solids presumably are determined by rates of diffusion of the pertinent ions in the solids. Little is known about these diffusion rates for the type of micro-crystalline or amorphous materials with which we are dealing. However, the extreme rapidity of the reactions of Ag(I) with most sulfides and their large extent are probably in part connected with the large self-diffusion coefficient of Ag(I) in Ag_2S. PESCHANSKI[22] has reported it to be *ca.* $2 \cdot 10^{-9}$ cm^2sec^{-1} at 25°. This value is large enough to allow half times of reaction of the order of seconds with particles in the micron range provided that this diffusion step is rate determining. How far this is true cannot be established at present.

SUMMARY

Sulfides of Ag(I), Fe(II), Cu(II), Zn(II), Pb(II), Cd(II) and As(III) "adsorb" a number of transition metal ions from solution. The process proceeds primarily through metathetical reactions in which the metal of the sulfide is displaced by appropriate ions in solution. The reactions are usually fast and can be carried out in

small columns at flow rates of several cm/min. With Ag(I) and 1- to 2-cm columns of various sulfides, flow rates as high as 50 cm/min were feasible without breakthrough. The percent conversion of the sulfides at 50 % breakthrough varied from 5 % for the reaction of Hg(II) with CuS at 25° to about 128 % for the reaction of Hg(II) with Ag_2S. With ZnS uptakes as high as 20 moles of Ag(I) per liter of bed were obtained at 50 % breakthrough and a flow rate of 7 cm/min. While possibility of conversion must depend on relative stability of the sulfides, neither the rate of reaction nor the completeness of conversion seems related to the relative solubilities. The nature of the solid and the ease with which diffusion takes place are presumably important factors.

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THE SEPARATION OF THE LANTHANIDES AND YTTRIUM BY CATION EXCHANGE ELUTION WITH AMMONIUM α-HYDROXYISOBUTYRATE AND LACTATE

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INTRODUCTION

The use of ammonium α-hydroxyisobutyrate solutions for rare earth separations has been discussed by several investigators[1–5]. Comparison of the relative separation factors with other eluants indicates that this eluant is so far the best[2].

The degree of separation for an experimental arrangement depends on variables such as temperature, type and size of the cation exchange resin particles, flow rate of the eluting agent, dimensions of the column, but for a given eluting agent the relative position of the various rare earth metals remains approximately constant. However, no attention has been given to the influence on the separation degree of the addition of an indifferent electrolyte to the eluant. The approach has been to utilize the data from elutions with and without addition of an indifferent electrolyte (in this case ammonium perchlorate), to obtain more information on the complexes present in the effluent solutions.

Since preliminary experiments indicated that the separation at 80° was not much better than at room temperature, these investigations were made at 25.0° ± 0.1°. Only trace quantities of the lanthanides were used.

EXPERIMENTAL

Reagents and apparatus

Ion exchange resin. Dowex 50-WX 8, 200–400 mesh, was washed twice with 6 *M* HCl, to remove iron, and afterwards with distilled water. The colloid particles were decanted off. The resin was converted into the ammonium form with 6 *M* ammonium hydroxide, washed and stored in distilled water until the column was loaded.

Elution agent. Aqueous solutions of α-hydroxyisobutyric acid (Fluka) and lactic acid (BDH) were adjusted to a fixed pH value by addition of concentrated ammonium hydroxide. Commercial lactic acid often exists partially as a lactone dimer[2]. To convert it completely to the free acid form, an aqueous solution of the lactic acid was neutralized with sodium hydroxide. The acid was regenerated by passing the solution through a bed of cation exchange resin in the hydrogen form where the sodium ions are replaced by hydrogen ions.

Tracers. The lanthanide isotopes used were obtained by neutron irradiating

of the Johnson, Matthey & Co. "spec-pure" oxides in the BR-1 reactor at a flux of 10^{12} n·sec^{-1}·cm^{-2}.

Column. Resin columns of about 3 mm diameter and 15–20 cm in length were used. The column was surrounded by a water jacket and thermostated at 25.0° ± 0.1°.

Registration apparatus. The experiments were carried out using an apparatus similar to the one described by SPEECKE AND HOSTE[6].

Procedure

Column experiments. The resin column was packed by pouring an aqueous slurry of the resin, previously degassed, into the column. Before each experiment, the column was washed with the eluant until the pH of the effluent and eluant were identical. The lanthanide tracers were gently placed on the top of the column and the eluant was carefully introduced to start elution. A flow rate of approximately 0.75 ml·cm^{-2}·min^{-1} was used in all runs.

The distribution coefficients were calculated according to the method described by KRAUS AND MOORE[7,8].

Equilibrium experiments. The distribution coefficients K_D were also determined by equilibrium studies under conditions similar to the column experiments. K_D is defined by the expression[6]:

$$K_D = \frac{\text{activity/g resin}}{\text{activity/ml solution}} = \frac{S - E}{E} \cdot \frac{\text{ml}}{\text{g}} \qquad (1)$$

where: S = activity of the solution before equilibration,

E = activity of the solution after equilibration with the resin.

The solutions of the ligand and the tracer were added in 50 ml conical flasks. Resin portions of 100 mg were weighed accurately and transferred. The flasks were agitated for 15 h in a constant temperature bath at 25.0 ± 0.1°.

Identification. The lanthanide isotopes in the effluent were identified by taking γ-spectra with an Intertechnique 400 S.A. multichannel spectrometer or by following the half-lives by γ-counting, using a NaI(Tl) well-type scintillation detector coupled to a Tracerlab superscaler.

RESULTS AND DISCUSSION

Fig. 1 and Fig. 2 show the distribution coefficients *versus* pH, using solutions of the ammonium salt of α-hydroxyisobutyric acid as eluant, 0.1 and 0.3 M respectively, without the indifferent electrolyte.

Since the concentration of the isobutyrate ion determines the elution volume and thus the distribution coefficients, these are plotted in Fig. 3 against the pL = −log [L$^-$] values. The anion concentration at each pH and molarity was calculated from the ionization constant of the α-hydroxyisobutyric acid: $K_A = 1.29 \cdot 10^{-4}$ and $1.33 \cdot 10^{-4}$ respectively for 0.1 M and 0.3 M of the acid[9]. The results using 1 M ammonium salt solutions of lactic acid as eluting agent are represented in Fig. 4.

The influence of increasing ionic strength on the distribution coefficient is shown in Table I. The K_D values are determined for approximately the same [L$^-$] values using increasing amounts of ammonium perchlorate. The data for the ionization constants (column 4) are given elsewhere[9].

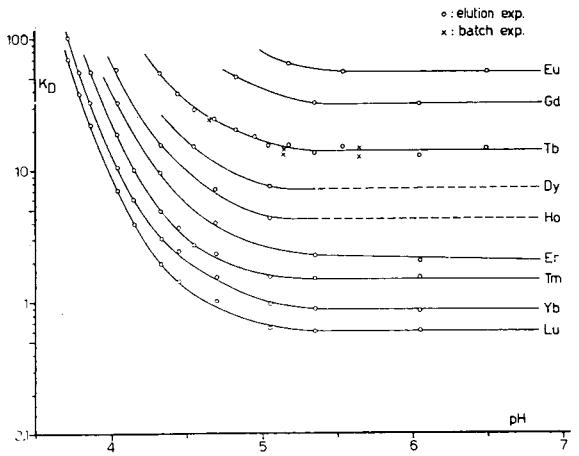

Fig. 1. Distribution coefficients *vs.* pH using 0.1 *M* ammonium α-hydroxyisobutyrate without indifferent electrolyte.

Since the stability constants of the lanthanides have been determined with α-hydroxyisobutyrate and lactate ligands in 0.2 *M* sodium perchlorate solution, a series of elution experiments in ammonium perchlorate was also performed. The results of these elutions (with 0.2 *M* ammonium perchlorate and 0.1 *M* α-hydroxyisobutyric acid) are plotted in Fig. 5.

Some points in Figs. 1 and 5 were checked by equilibrium experiments.

Because uni-negative tetra-ligand complexes are formed between the trivalent lanthanide ions and the α-hydroxyisobutyrate or lactate ions, the distribution coefficient is defined by:

$$K_D = \frac{\sum [\mathrm{M}]_{\text{resin}}}{\sum [\mathrm{M}]_{\text{solution}}} = \frac{[\mathrm{M}^{3+}]_R + [\mathrm{ML}^{2+}]_R + [\mathrm{ML}_2^{+}]_R}{[\mathrm{M}^{3+}] + [\mathrm{ML}^{2+}] + [\mathrm{ML}_2^{+}] + [\mathrm{ML}_3] + [\mathrm{ML}_4^{-}]} \qquad (2)$$

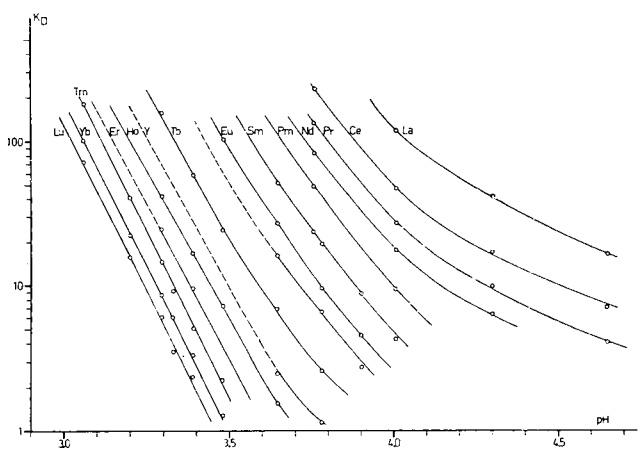

Fig. 2. Distribution coefficients *vs.* pH using 0.3 *M* ammonium α-hydroxyisobutyrate without indifferent electrolyte.

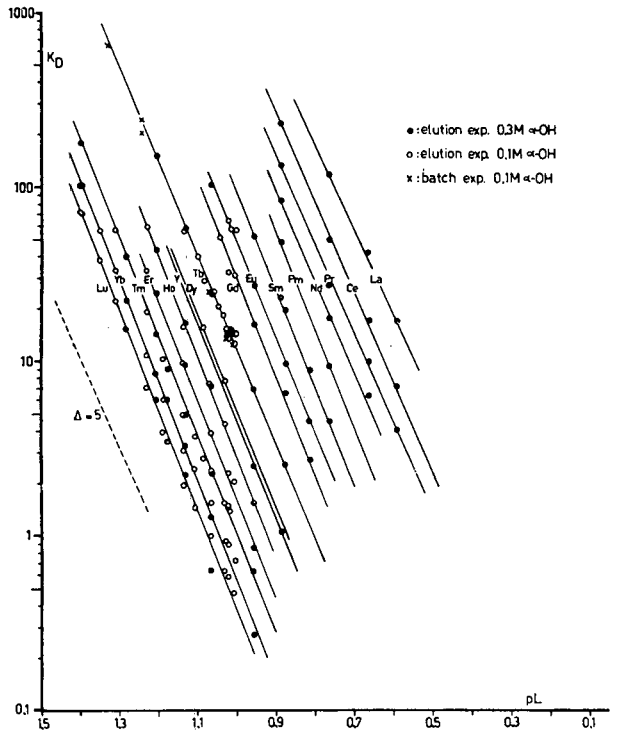

Fig. 3. Distribution coefficients *vs.* α-hydroxyisobutyrate concentration, without indifferent electrolyte.

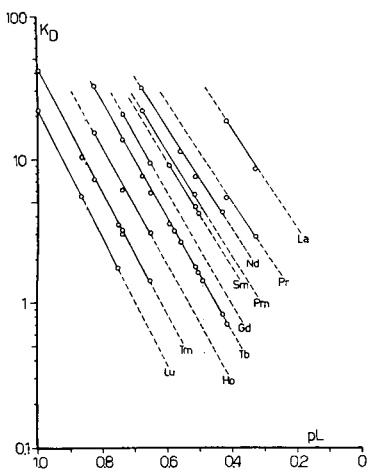

Fig. 4. Distribution coefficients *vs.* lactate concentration, without indifferent electrolyte.

TABLE I

INFLUENCE OF THE IONIC STRENGTH ON THE DISTRIBUTION COEFFICIENTS OF THE THULIUM–α-HYDROXYISOBUTYRATE COMPLEX SYSTEM

Moles NH_4ClO_4	*Moles acid*	*pH*	pK_A	$[L^-]$	I_{total}	K_D
—	0.1	3.16	3.885	0.0158	0.016	29,060
0.001	0.1	3.16	3.88	0.0160	0.017	25,815
0.01	0.1	3.16	3.875	0.0162	0.026	9,727
0.01	0.3	2.62	3.865	0.0161	0.026	8,510
0.025	0.1	3.12	3.865	0.0152	0.040	3,352
0.05	0.1	3.16	3.845	0.0171	0.067	1,057
0.075	0.3	2.57	3.82	0.0160	0.091	471
0.1	0.1	3.12	3.81	0.0169	0.117	280
0.1	0.3	2.55	3.80	0.0160	0.116	260
0.2	0.1	3.12	3.80	0.0174	0.217	35

However, in an investigation[10] of some lanthanide–α-hydroxyisobutyrate complexes using FRONAEUS' cation exchange method[11] it was shown that the adsorption of positively charged complexes on the resin is very uncertain in the examined $[L^-]$-concentration range, amongst other reasons because the adsorption of $M^{3+} \gg ML^{2+} \gg ML_2^+$.

Moreover these experiments were carried out in such a pL range that, according

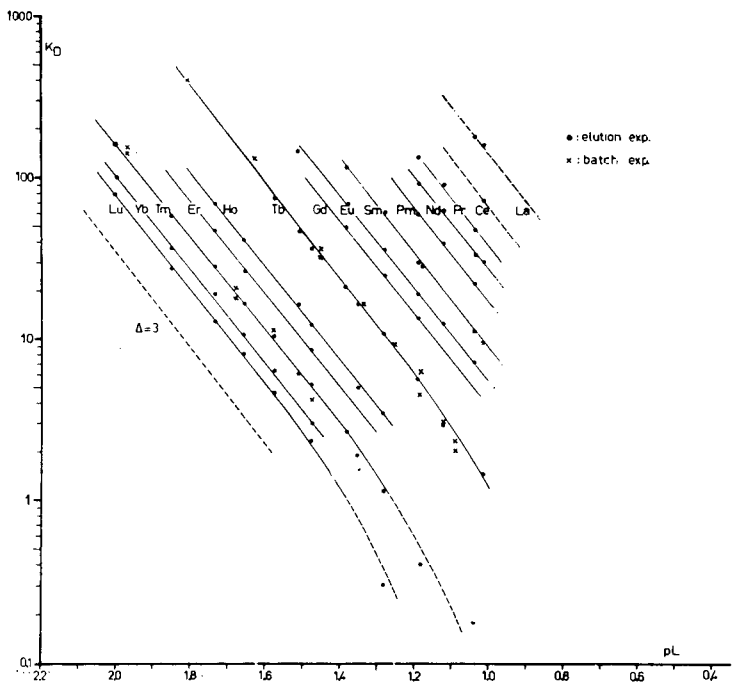

Fig. 5. Distribution coefficients *vs.* α-hydroxyisobutyrate concentration, in presence of 0.2 *M* ammonium perchlorate.

to the distribution of the complexes calculated from the stability constants, mainly ML_3 and ML_4^- complexes are formed[12]. Consequently equation (2) becomes in the pL range investigated:

$$K_D \cong \frac{[M^{3+}]_R}{[M^{3+}] + [ML^{2+}] + [ML_2^+] + [ML_3] + [ML_4^-]} \quad (3)$$

Substituting ML^{2+}, ML_2^+,... by the respective stability constants:

$$\beta_n = \frac{[ML_n^{3-n}]}{[M^{3+}]\,[L^-]^n} \quad (4)$$

gives:

$$K_D \cong \frac{[M^{3+}]_R}{[M^{3+}]\left(1 + \sum_{n=1}^{n=4} \beta_n [L^-]^n\right)} \quad (5)$$

If one species is predominant in solution, the slope of a log–log plot is given by:

$$\frac{\mathrm{d} \log K_D}{\mathrm{d} \log [L^-]} = -n \quad (6)$$

This slope gives in fact the average number of ligands bound per metal ion. The value of d log K_D/d log $[L^-]$ in Fig. 3 is approximately —5 between the pL values from 0.5 to 1.4, which would lead to the assumption that the complexes in the solution present would be of the form ML_5^{2-}. These doubly charged complexes ought to be excluded from a consideration of steric factors[13] and also from calculations using the stability constants determined by potentiometric titration[12]. The deviation of the slope from —4 (complexes ML_4^-) in the absence of indifferent electrolyte is probably due to the fact that the activity coefficients do not remain constant as an effect of changing ionic strength in function of pL. The slope found by SMITH AND HOFFMAN[3] using 0.5 *M* and 1 *M* solutions of the ammonium salt of α-hydroxyisobutyric acid as eluant is of the same order. HOLM and coworkers[13] found also by anion exchange and electromigration experiments in 0.3–0.5 *M* acid that ML_4^- complexes are predominant.

Column experiments using 1 *M* ammonium lactate solutions without indifferent electrolyte (Fig. 4) give a d log K_D/d log [L] plot with a negative slope also larger than 4, between the pL values from 0.3 to 1.0, indicating the existence of ML_4^- complexes.

However, the composition of the complexes determined from column experiments using α-hydroxyisobutyrate and lactate solutions at a constant ionic strength agrees with the composition derived from potentiometric data. The slope of the log–log plot (Fig. 5) is approximately —3 in the pL range between 1.0 and 2.0. Essentially ML_3 complexes are formed, as can also be calculated from the stability constants[12]. This is also proved by calculating the stability constants β_3 from the experimental K_D values[10] using SCHUBERT's method[14]. These constants are in good agreement with those determined by potentiometric titration[12].

A summary of the separation of the lanthanides and yttrium is reproduced in Table II. For each eluant, column A gives the separation factor of two adjacent lanthanides with atomic numbers Z and $Z + 1$: $K_D{}^Z/K_D{}^{Z+1}$. Column B gives the K_D values relative to gadolinium with $K_D{}^{Gd} = 1.0$.

The values of column B and also the separation factors calculated from the stability constants are plotted *versus* the atomic numbers in Fig. 6. The separation factor of two adjacent lanthanides, x and y, is given by:

$$\alpha_y{}^x = \frac{K_{D_0}{}^x \cdot \beta_n{}^y}{K_{D_0}{}^y \cdot \beta_n{}^x} \cong \frac{\beta_n{}^y}{\beta_n{}^x} \qquad (7)$$

where $\beta_n{}^x$ and $\beta_n{}^y$ are the stability constants of the complexes involved. According to POWELL AND SPEDDING[15] the ratio of the K_{D_0} values for adjacent pairs of lanthanides would be less than 1.08. Similar conclusions can be drawn from distribution data of SURLS AND CHOPPIN[16]. The constants β_3 and β_4 were used in the calculations,

TABLE II

SEPARATION EFFICIENCY OF THE LANTHANIDES AND YTTRIUM BY CATION EXCHANGE ELUTION WITH α-HYDROXYISOBUTYRATE AND LACTATE SOLUTIONS

Element	*α-Hydroxyisobutyrate*				*Lactate*	
	With 0.2 M NH_4ClO_4		*Without NH_4ClO_4*		*Without NH_4ClO_4*	
	A	*B*	*A*	*B*	*A*	*B*
Lu		0.0275		0.013		0.070
	1.345		1.54		—	
Yb		0.037		0.020		—
	1.57		1.70		—	
Tm		0.058		0.034		0.15
	1.66		1.71		—	
Er		0.096		0.058		—
	1.47		1.73		—	
Ho		0.141		0.100		0.335
	—		1.80		—	
Dy		—		0.180		—
	—		2.30		—	
Tb		0.45		0.42		0.69
	2.23		2.40		1.45	
Gd		1.00		1.00		1.00
	1.40		1.65		—	
Eu		1.40		1.65		—
	1.64		1.88		—	
Sm		2.30		3.10		1.60
	1.92		2.13		1.19	
Pm		4.40		6.60		1.90
	1.59		1.54		1.40	
Nd		7.0		10.15		2.70
	1.43		1.68		1.70	
Pr		10.0		17.00		4.60
	1.75		1.85		—	
Ce		17.5		31.50		—
	2.06		1.97		—	
La		36.0		62.00		14.26
Y		—		0.16		—

respectively for the experiments with 0.2 M and without ammonium perchlorate.

The best eluant, as far as separation factors are concerned, is the one whose curve has the greatest slope. The improvement obtained with α-hydroxyisobutyrate as eluant in regard to lactate solutions is a result of the increase in complex stability

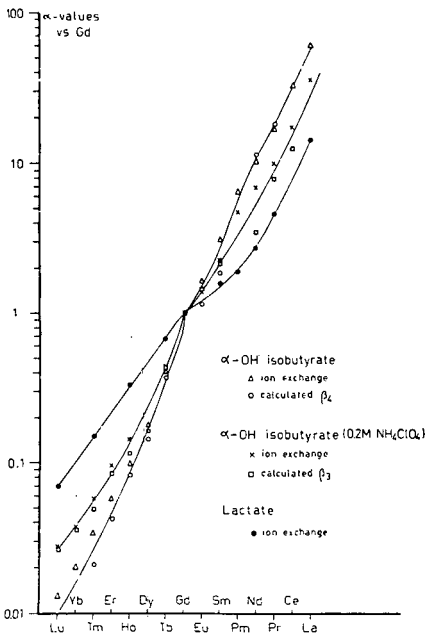

Fig. 6. Elution position of the lanthanides relative to gadolinium, determined by ion exchange measurements and by calculation from the stability constants of the respective complexes.

due to the increase of the inductive effect of the substitution of hydrogen by a methyl group in the α-carbon position[17]. Further introduction of larger alkyl groups does not seem to improve the separation efficiency[18].

The addition of ammonium perchlorate appears to have a definite adverse effect on the separation. As practical separation of the lanthanides is only possible for K_D values less than approximately 200, it is necessary to perform elution experiments at higher pL ranges, when working at higher ionic strengths. Consequently there are less ligands bound per metal ion and the apparent stability constants, and also the separation factors α, decrease. In solutions without ammonium perchlorate the elutions are performed in a pL range approximately from 0.5 to 1.3 where mainly ML_4^- complexes occur, and in solutions with 0.2 M ammonium perchlorate added in a pL range approximately from 1.0 to 2.2 where mainly ML_3 complexes are formed.

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SUMMARY

The separation of the lanthanides and yttrium by cation exchange elution from Dowex-50 WX resin columns with α-hydroxyisobutyrate and lactate solutions has been investigated with and without addition of ammonium perchlorate. Addition of an indifferent electrolyte to the eluant decreases the practical separation efficiency. This phenomenon can be explained from complex formation data. From the variation of the logarithm of the distribution coefficient as a function of the logarithm of the ligand concentration, $[L^-]$, in absence of an indifferent electrolyte, no valid conclusion can be drawn concerning the complexes formed in solution, contrary to the measurements in presence of an indifferent electrolyte.

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A SPECIFIC METHOD FOR THE SEPARATION OF RHENIUM BY ANION EXCHANGE CHROMATOGRAPHY

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Systematic investigations on the ion exchange behavior of metals in thiocyanate media are rather scarce[1-6]. We have shown that the distribution coefficient of Re(VII) on Dowex 1 in thiocyanate–chloride media exhibited a rather unique feature: the K_d value has a minimum at 0.5 M NH_4SCN and increases at both higher and lower concentrations of NH_4SCN with a fixed concentration of HCl (0.5 M HCl)[7]. This behavior of Re(VII) provided a basis for quantitative anion exchange separation of Re(VII) from Mo(VI) and Tc(VII), which are strongly adsorbed on the column under the same conditions. Although there are several available methods for the separation of Re(VII)[8,9], a specific method is still lacking. This work has been extended in a more comprehensive manner and detailed results are presented below on the separation of Re(VII) from a considerable number of metal ions as well as Mo(VI) and Tc(VII) using a NH_4SCN–HCl eluent with an anion exchange column.

EXPERIMENTAL

Apparatus, reagents and solutions

All chemicals used were of analytical reagent grade, unless otherwise stated. A strong base type anion exchanger, Dowex 1, X-8, 100–200 mesh particle size, in the thiocyanate form, was used. The pretreatment of the resin and the column preparation are the same as those described in the previous paper[7]. The resulting resin bed was 1.0 cm in diameter and 6.5 cm long. Stock solutions of metal ions were prepared by dissolving appropriate amounts of the chloride, oxide or carbonate of the metals in HCl and diluting to a definite volume to give 10 mg metal ions per ml of 0.5 M HCl. Two exceptions are W(VI) and V(V) solutions, which are prepared from $Na_2WO_4 \cdot 2H_2O$ and NH_4VO_3, respectively. Further dilution of the stock solution was made whenever necessary with 0.5 M HCl. Triplicate determinations were performed using an appropriate analytical method to standardize each stock solution. The Sn(IV), Sb(III) and Bi(III) solutions were unstable at the concentration of free acid employed (0.5 M HCl). They were stabilized by adding more acid to give a free acid concentration of 3 M HCl.

Procedure

Before use the resin bed should be washed thoroughly with several column

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volumes of 0.5 *M* NH_4SCN–0.5 *M* HCl solution. The sample solution is adjusted to approximately 10 ml, which is 0.5 *M* in NH_4SCN and 0.5 *M* in HCl. The samples is then placed on the column dropwise. When the sample solution almost reaches the top of the column bed, it is eluted with 0.5 *M* NH_4SCN–0.5 *M* HCl solution at a flow rate of 1 ml/min, and the effluent is collected. The removal of non-adsorbable elements listed in Table II is complete in the first 25 ml of effluent. Elution is continued with the same eluent to remove Re(VII). Re(VII) is completely recovered in the next 40 to 150 ml of effluent. The remaining elements are stripped from the column with the appropriate amount of eluent listed in Table IV. The methods employed for the analysis of the effluent are summarized in Table I.

TABLE I

ANALYTICAL METHODS USED

Cation	*Method*
Re(VII)	Colorimetrically with KSCN–$SnCl_2$
Li(I), Na(I), K(I)	Flame-photometrically
Be(II)	Colorimetrically as complex with 8-hydroxyquinoline
Zn(II), Cd(II), Mg(II), Mn(II)	Titration with EDTA using Eriochrome Black T as indicator
Hg(II)	Colorimetrically with Dithizone
Cu(II), Ca(II), Ni(II), Co(II)	Titration with EDTA using Murexide as indicator
Ba(II)	Titration with EDTA using Pyrocatechol Violet as indicator
Y(III), Ce(III), Eu(III), Dy(III), Er(III), Th(IV), Bi(III)	Titration with EDTA using Xylenol Orange as indicator
Al(III), In(III), Ga(III)	Titration with EDTA using Cu-Pan* as indicator
Zr(IV)	Colorimetrically with Alizarin S
Ge(IV)	Colorimetrically with Phenylfluorone
Sn(IV), W(VI)	Colorimetrically with Dithiol
V(V)	Colorimetrically with sodium tungstate
As(III)	Colorimetrically with ammonium molybdate
Sb(III)	Colorimetrically with potassium iodide
Cr(III)	Colorimetrically using its characteristic absorption band at 610 mμ
Se(IV), Te(IV)	Colorimetrically as metallic colloid after reduction with stannous chloride
Fe(III)	Titration with EDTA using Variamine Blue B as indicator
Ru(III)	Colorimetrically with thiourea
U(VI)	Colorimetrically with hydrogen peroxide

* Cu-Pan is a mixture of 1-(2-pyridylazo)-2-naphthol and Cu–EDTA.

RESULTS AND DISCUSSION

The study of the elution behavior of individual metal ions indicates that Li(I), Na(I), K(I), Be(II), Mg(II), Ca(II), Ba(II), Ge(IV), As(III), Cr(III), Al(III), Y(III), Ce(III), Eu(III), Dy(III), and Er(III) do not show any marked adsorption from the 0.5 *M* NH_4SCN–0.5 *M* HCl solution, so that they are completely recovered from the

column in the first 25 ml fraction of the effluent. Quantitative data for actual separations using the 0.5 *M* NH_4SCN–0.5 *M* HCl eluent are given in Table II.

With the elution conditions above, the greater part of the Te(IV), amounting to 75 %, comes through from the sample solution, the remainder being retained on the resin. Thus Re(VII) can be quantitatively separated from Te(IV). However, the

TABLE II

QUANTITATIVE SEPARATION OF Re(VII) FROM FOREIGN METAL IONS ON ANION EXCHANGE COLUMNS

Foreign ions	*Taken (mg)*		*Recovered (mg)*	
	Re	*Foreign ions*	*Re*	*Foreign ions*
Li(I)	2.00	1.51	1.93	1.51
Na(I)	2.00	10.0	1.97	10.0
K(I)	2.00	10.0	2.05	10.0
Be(II)	2.00	4.00	1.93	4.11
Mg(II)	1.00	20.0	1.07	19.5
Ca(II)	1.00	20.0	1.08	19.2
Ba(II)	1.00	20.0	1.04	20.3
Al(III)	2.00	8.50	2.04	8.43
Y(III)	1.00	2.40	1.03	2.33
Ce(III)	2.00	7.70	1.99	7.67
Eu(III)	2.00	9.82	2.02	9.50
Dy(III)	2.00	9.10	2.01	8.97
Er(III)	2.00	9.85	2.09	9.65
Cr(III)	2.00	10.0	1.90	10.0
As(III)	2.00	4.00	2.05	3.94
Ge(IV)	2.00	1.00	2.14	0.99
Te(IV) (tellurite)	2.00	4.00	2.01	3.95
Se(IV) (selenite)	2.00	20.0	2.02	19.9

partial retention of Te(IV) on the column can be avoided by decreasing the concentration of NH_4SCN down to 0.05 *M*, at a fixed concentration of HCl (0.5 *M*). Contrary to Te(IV), Se(IV) is strongly adsorbed by the resin from the 0.5 *M* NH_4SCN–0.5 *M* HCl solution. Where Se(IV) is adsorbed there is actually no adequate eluting agent to remove it effectively from the column. Fortunately the adsorbability of Se(IV) on the resin decreases rapidly with decreasing concentration of NH_4SCN, approaching zero at 0.025 *M* NH_4SCN at the fixed concentration of HCl (0.5 *M*). Therefore Se(IV) can be quantitatively collected in the first 25 ml fraction of the effluent, while Re(VII) is retained on the top of the column under the same conditions. Afterwards Re(VII) is successfully eluted with 0.5 *M* NH_4SCN–0.5 *M* HCl.

Mn(II), Ni(II), Th(IV) and Zr(IV) do not separate from Re(VII) clearly with the elution conditions given in the above procedure. Certain amounts of these elements come through in the same fraction as Re(VII). This difficulty can be overcome by decreasing the concentration of NH_4SCN to 0.025 *M*, keeping the HCl concentration constant at 0.5 *M* so that these metal ions show practically no adsorption on the resin.

For each pair of elements, a profile elution curve was obtained by collecting fractions and determining the metal content of each. The profile curves thus obtained

are illustrated in Fig. 1 for the pairs Re(VII)–Mn(II) and Re(VII)–Th(IV). Results of the quantitative separations of Re(VII) from Th(IV), Zr(IV), Ni(II), and Mn(II) are given in Table III.

The curves in Fig. 1 show that there is essentially no tailing of bands.

TABLE III

QUANTITATIVE SEPARATION OF Re(VII) FROM Th(IV), Zr(IV), Ni(II), AND Mn(II) ON ANION EXCHANGE COLUMNS

Foreign ions	*Taken (mg)*		*Recovered (mg)*	
	Re	*Foreign ions*	*Re*	*Foreign ions*
Mn(II)	2.00	5.34	1.94	5.25
Th(IV)	2.00	7.25	2.07	7.25
Ni(II)	1.00	5.02	1.03	5.20
Zr(IV)	2.00	9.95	2.04	9.65

Almost all the ions which form thiocyanate complexes are strongly retained on the top of the resin bed. Thus the separation of Re(VII) from most of the metals belonging to the thiocyanate group will be sharp. Data on the separation of synthetic mixtures of Re(VII) and other individual metal ions are quoted in Table IV under the condition stated in the procedure. Because of the strong adsorbabilities of the elements listed in Table IV from the thiocyanate–chloride medium there are some difficulties in the choice of a suitable eluent for the successful elution of individual metal ions.

Eluents finally found suitable for the successful elution of different cations are given in Table IV along with the data on the separation. Re(VII) can be separated with good results from any of the separated ions which are present in high proportions relative to Re(VII). Sn(II) caused difficulty at first in the elution of Re(VII) so that

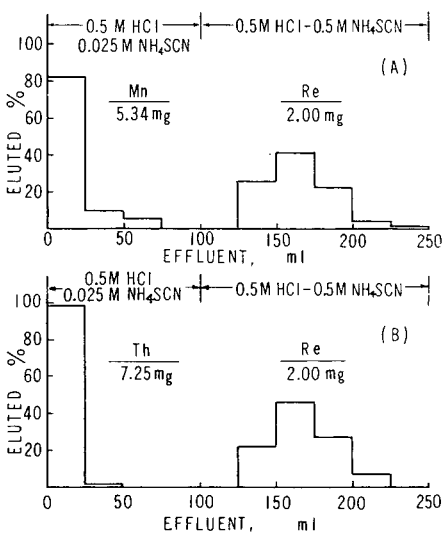

Fig. 1. Profile curves of separations of Re(VII)–Mn(II) and Re(VII)–Th(IV).

TABLE IV

SEPARATION OF RHENIUM FROM FOREIGN METAL IONS

Re (mg)		*Foreign ions (mg)*			*Eluent used for metal ions*
Taken	*Found*		*Taken*	*Found*	
2.00	1.92	Ga(III)	9.68	9.78	Ga quantitatively eluted with 150 ml of 0.5 *M* H_2SO_4
2.00	1.88	In(III)	8.64	8.64	In quantitatively eluted with 275 ml of 0.5 *M* HNO_3
2.00	1.98	Sb(III)	2.32	2.35	Sb quantitatively eluted with 100 ml of 0.5 *M* H_2SO_4
2.00	2.04	W(VI)	4.00	4.00	W quantitatively eluted with 25 ml 0.5 *M* NaOH–0.5 *M* NaCl
2.00	1.98	Bi(III)	4.45	4.50	Bi quantitatively eluted with 125 ml of 0.5 *M* H_2SO_4
2.00	1.95	Fe(III)	4.94	5.60	Fe quantitatively eluted with 300 ml of 0.5 *M* HNO_3
1.00	0.94	U(VI)	5.10	4.74	93% uranyl recovered by elution with 250 ml of 1 *M* HCl
2.00	2.00	Sn(IV)	2.95	2.93	Sn quantitatively eluted with 25 ml of 0.5 *M* NaOH–0.5 *M* NaCl
2.00	1.98	V(V)	4.74	4.73	V quantitatively eluted with 150 ml of 2 *M* NaOH
2.00	2.02	Cu(II)	4.94	4.90	Cu quantitatively eluted with 125 ml of 0.5 *M* NH_4OH–0.5 *M* NH_4Cl
1.00	1.04	Zn(II)	11.0	10.0	Zn quantitatively eluted with 100 ml of 1 *M* $HClO_4$
1.00	1.07	Cd(II)	10.5	10.6	Cd quantitatively eluted with 100 ml of 1 *M* $HClO_4$
1.00	0.96	Co(II)	5.10	5.20	Co quantitatively eluted with 100 ml of 1 *M* $HClO_4$

both Re(VII) and Sn(II) came through partially from the sample solution on elution with 0.5 *M* NH_4SCN–0.5 *M* HCl. Re(VII) failed to break through further in the subsequent fraction of the same effluent. This difficulty can be simply avoided by oxidizing Sn(II) to the tetravalent state with H_2O_2 prior to column separation. Sn(IV) shows strong adsorption on the column, but can be easily stripped by elution with 0.5 *M* NaCl–0.5 *M* NaOH solution, as indicated in Table IV.

The separation of Re(VII) from Hg(II) and Ru(III) is complete with the procedure employed. However, considerable difficulty was encountered in stripping Hg(II) and Ru(III), which remain in a band near the top of the column. We have not yet found any suitable eluting system to remove them from the column.

The flow rate of the eluent has a considerable effect on the shape of the elution band of Re(VII). At a flow rate of 0.5 ml/min the total volume of eluent (0.5 *M* NH_4SCN–0.5 *M* HCl) required to strip Re(VII) from the column was 60 to 70 ml, about half of that obtained at a flow rate of 1 ml/min.

To study the effect of the cross-linking of the resin on the elution behavior of Re(VII), Dowex 1, X-4, X-8, and X-10 were selected to obtain elution profile curves. With higher cross-linking, Re(VII) tends to break through in the later fraction of effluent. However, there is actually no difference in the complete elution amongst the resin types employed.

In Fig. 2 the effect of cross-linking on the shape of the elution band is illus-

trated. It can also be seen that the elution band using the resin of lower cross-linking tends to show negative skewness, while that using a resin of higher cross-linking shows positive skewness. It is worth noting here that it was possible in the previous work[7] to strip Re(VII) from the Dowex 1, X-8 column of the same size as employed here by eluting at the flow rate of 1 ml/min with approximately 40 ml of 0.5 *M* NH_4SCN–0.5 *M* HCl, which is almost less than half that required in this work.

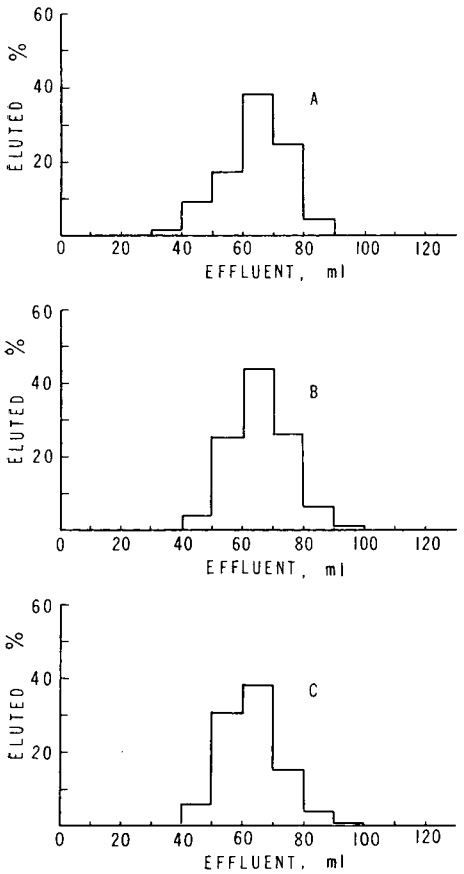

Fig. 2. Effect of cross-linking on the elution behavior of Re(VII). (A) Dowex 1, X-4, 200–400 mesh, flow rate 0.5 ml/min; (B) Dowex 1, X-8, 200–400 mesh, flow rate 0.5 ml/min; (C) Dowex 1, X-10, 200–400 mesh, flow rate 0.5 ml/min.

Although an explanation of this point cannot be given at present, it seems likely that a change in resin lot would have a considerable effect on the elution band width of Re(VII).

SUMMARY

A systematic survey of the adsorbabilities of cations with a strong base type resin, Dowex 1, X-8, in a thiocyanate–hydrochloric acid system indicates that the

difference between the distribution coefficient of Re(VII) and those of a considerable number of other ions is large enough for good separation. This fact provides the means of developing an anion exchange chromatographic procedure for the separation of Re(VII) from other ions. Re(VII) is eluted with 0.5 *M* NH_4SCN–0.5 *M* HCl, while alkali metals, alkaline earth metals, rare earths, Y(III), Cr(III), Al(III), As(III) and Ge(IV) are eluted earlier in the effluent from the sample solution. Ions forming thiocyanate complexes such as Zn(II), Cd(II), Hg(II), Cu(II), In(III), Ga(III), Sn(IV), V(V), Sb(III), Sb(V), Bi(III), W(VI), Fe(III), Co(II), Ru(III) and U(VI), are retained on the column. An effective eluting system for these ions is also given. Separations of Re(VII) from 38 metal ions are reported.

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Short Communication

Methylated Sephadex as support in reversed phase partition chromatography

A number of different supports for the stationary phase have been used in reversed phase chromatography, *e.g.* rubber[1], siliconized kieselguhr[2], acylated or siliconized cellulose[3,4] and polyethylene powder[5]. During a study of the use of methylated Sephadex in gel filtration of lipids and lipid-soluble compounds[6] we also tested the ability of this material to carry a non-polar stationary phase in reversed phase partition chromatography. This Sephadex derivative was found to be very useful for solvent systems of medium polarity. The present paper describes this property using bile acids as model substances for the separations. Some preliminary results have been reported[7].

Experimental

Sephadex G 25, fine, in bead form, was purchased from AB Pharmacia, Uppsala, Sweden, and was methylated with dimethyl sulfate in alkali[8]. The content of methoxyl groups was 35.2–36.5 %.

The solvent systems tried were those used previously for bile acid separations[9]. Solvent system F 2 consisted of methanol–water–chloroform–heptane (180:120:45:5, v/v) and solvent system C consisted of methanol–water–chloroform–isooctanol (150:150:15:15).

Six ml of the stationary phase were mixed thoroughly with 4.5 g dried methylated Sephadex. Twenty-five ml of the mobile phase were added and the resulting slurry was poured into a chromatography tube having a diameter of 12 mm. After light homogenization with a perforated plunger the column was allowed to settle by gravity with free solvent flow. The sample was dissolved in 2 ml of the mobile phase and applied to the top of the column. When the sample had been rinsed into the column, mobile phase was added and 1–4 ml fractions collected. The flow rate was 20–40 ml/h. The fractions were titrated with 0.02 *N* methanolic NaOH.

When the chromatography was completed the column packing was emptied into a sintered glass funnel and was washed with a suitable solvent (*e.g.* ethanol). After drying at about 50° the methylated Sephadex can be used again.

Results and discussion

The capacity of methylated Sephadex to carry the stationary phase was tested by packing the support in a column with a large excess of stationary phase and then rinsing with mobile phase until no droplets of stationary phase appeared in the effluent. With the solvent systems tested, 4.5 g methylated Sephadex could carry 6 ml of stationary phase.

Fig. 1 shows the separation of cholic and deoxycholic acids with phase system F 2. The fractions were collected on a time basis. It was observed that the solvent flow decreased during the chromatography. This may be due to a slow change in the

composition of the stationary phase. No stationary phase, however, appeared as a separate phase in the effluent. The separations were not affected by the change in solvent flow and could be easily reproduced provided that the columns were made as described above.

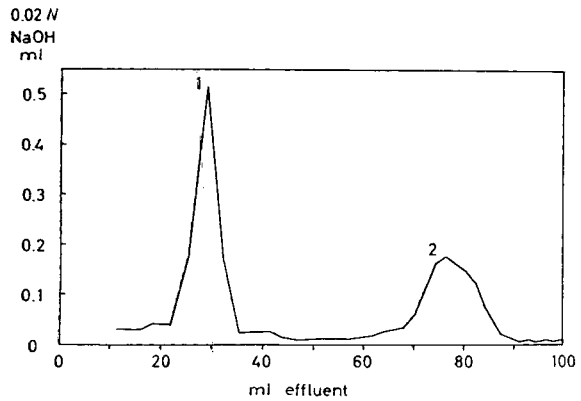

Fig. 1. Reversed phase partition chromatography of (1) cholic and (2) deoxycholic acids with phase system F 2, using methylated Sephadex G 25 as support for the stationary phase.

Glycocholic and cholic acids were separated with phase system C. With this system solvent flow changes were very small. Table I shows a comparison between effluent volumes on columns with siliconized Hyflo Supercel[9] and methylated Sephadex as support. It is seen that retention volumes are larger with the methylated Sephadex columns. In the case of phase system C this is probably due mainly to the fact that the methylated Sephadex can carry 50 % more stationary phase. With phase system F 2 it is possible that the stationary phase on the more polar methylated Sephadex has a lower proportion of heptane than the original solvent mixture causing a retardation of the bile acids. In contrast to siliconized Hyflo Supercel the methylated Sephadex cannot carry heptane as stationary phase and it does not swell in this solvent. It is therefore reasonable to assume that methylated Sephadex cannot be used as support in reversed phase chromatography with less polar solvent systems where the stationary phase causes little or no swelling of the Sephadex (*e.g.* benzene, toluene)[6].

TABLE I

COMPARISON BETWEEN SEPARATIONS OBTAINED ON COLUMNS WITH 4.5 g SILICONIZED HYFLO SUPERCEL CARRYING 4 ml STATIONARY PHASE AND COLUMNS WITH 4.5 g METHYLATED SEPHADEX G 25 CARRYING 6 ml OF STATIONARY PHASE

Bile acid	*ml effluent at peak fraction*			
	Phase system F 2		*Phase system C*	
	G 25	*Hyflo*	*G 25*	*Hyflo*
Glycocholic acid	—	—	45	35
Cholic acid	30	12	130	100
Deoxycholic acid	75	35	—	—

The titration values showed that quantitative recoveries of the bile acids were obtained.

The present study indicates that methylated Sephadex is a useful support in reversed phase chromatography with solvent systems of medium polarity. It is probably not suitable for systems less polar than F 2 used in this investigation but further experiments may show that it is of value in more polar solvents. Since the partially methylated Sephadex swells in water it might also be useful as a support for aqueous stationary phases in "straight" partition chromatography. The high capacity and the ease with which it can be regenerated are important advantages of this support.

Acknowledgement

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Notes

Separation of amino acid *n*-butyl esters by means of thin-layer chromatography

During previous work concerning the gas-chromatographic separation of amino acid derivatives, the purity of amino acid *n*-butyl esters was checked by means of thin-layer chromatography. The method used for the preparation of these esters is described elsewhere.[1]

Glass plates (20 × 20 cm) were covered with Kieselgel G (Merck) in layers of 0.25 mm. The solvent used for the separation of the butyl esters was a mixture of

benzene and *n*-butanol (75:25). Under these conditions only the esters migrate with a characteristic R_F value, while the amino acids do not move from the point of deposition.

The solvent system has been found useful for the separation of the following amino acid butyl esters: alanine, valine, leucine, isoleucine, norleucine, alloisoleucine, proline, allo-hydroxyproline, 4-hydroxyproline, threonine, glycine, methionine, aspartic, glutamic and α-aminobutyric acid.

TABLE I

R_F VALUES OF AMINO ACID BUTYL ESTERS IN THIN-LAYER CHROMATOGRAPHY

n-Butyl ester of	R_F		*Colours, after spraying with ninhydrin in 0.2% butanol*
	*A**	*B**	
Glycine	0.580	—	Orange
Alanine	0.540	—	Yellow
Allo-hydroxyproline	0.310	—	Yellow
α-Aminobutyric acid	0.300	—	Red
4-Hydroxyproline	0.271	—	Yellow
Norvaline	0.242	—	Purple-pink
Valine	0.200	—	Purple-pink
Norleucine	0.197	—	Red
Leucine	0.186	—	Red
Methionine	1.091	—	Pink
Isoleucine	0.188	—	Purple-pink
Glutamic acid	0.150	—	Orange
Phenylalanine	0.140	—	Pink-red
Aspartic acid	0.130	—	Orange
Citrulline	0.080	—	Orange
Lysine	0	0.340	Purple-pink
Histidine	0	0.360	Orange
Ornithine	0	0.355	Purple-pink

* Solvents: A = benzene–*n*-butyl alcohol (75:25, v/v). Running time: 1 h. B = *n*-butyl alcohol–acetic acid–water (120:30:50, v/v/v). Running time: 3h.

The esters of basic amino acids do not move with the above-mentioned solvent although their separation can be achieved using the common solvent system: *n*-butanol–acetic acid–water (120:30:50).

The R_F values of amino acid butyl esters on thin layers are reported in Table I.

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Prevention of adsorbent loss in thin-layer chromatography

Adsorbent layers of Silica Gel G sometimes slide off the plate below the solvent level, when water is a component of the developing system. KIRCHNER, MILLER AND KELLER[1] solved this problem by standing their glass strips containing the layer on a cotton wad saturated with solvent. We have tried this method with 20 cm × 20 cm plates and, while it aids in preventing the loss of the lower part of the layer, it tends to cause the solvent front to advance up the layer at an irregular rate, faster in some places and dipping in others. We have, therefore, devised another method of coping with this problem, which in our hands has proved more satisfactory.

We employ the Camag "Sandwich" Chamber method and equipment* for development of thin-layer chromatograms. The plate is spread with adsorbent in the usual manner. Prior to application of the sample the adsorbent layer is scraped off from a strip approximately 8 mm wide on all 4 sides of the plate (20 cm × 20 cm), as for two-dimensional chromatograms. The sample is then applied to the adsorbent layer. Two or three strips of white blotting paper, depending on the thickness of the blotting paper, are carefully placed across the bottom of the plate and the cardboard spacer is then placed, as is usual with this equipment, on the other three sides (Figs. 1 and 2). The blotting paper was extracted with three 5 min changes of acetone and

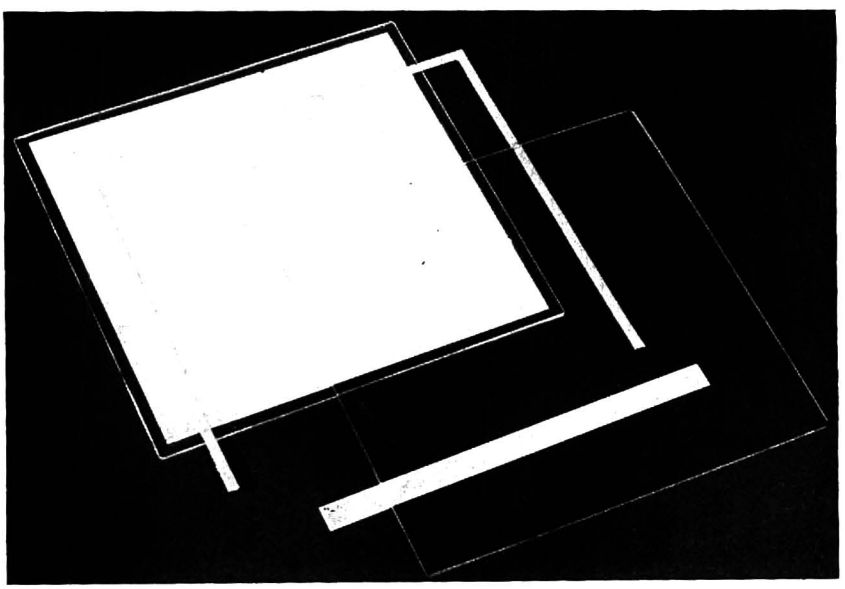

Fig. 1. Exploded view, showing blotting paper strips in relation to equipment.

air-dried before use. It is cut wide enough to overlap the bottom of the adsorbent layer by 2–3 mm. A second uncoated glass plate is then placed on top of the spacer and blotting paper and the two plates are clamped together to form a "sandwich" (Fig. 2). The "sandwich" is then placed in the solvent and development proceeds as

* Camag, Muttenz, Switzerland.

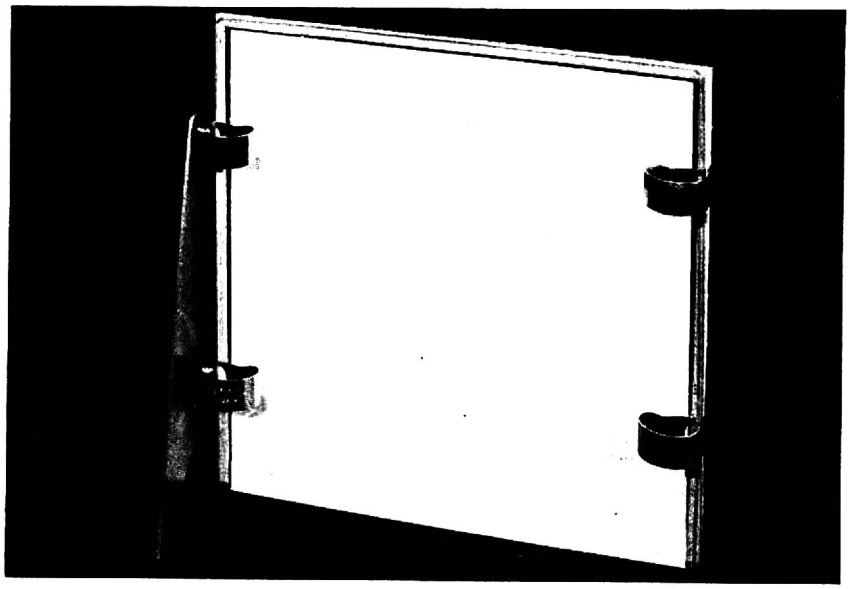

Fig. 2. Assembled equipment, showing blotting paper strips in place.

usual. The blotting paper acts as a barrier which holds back the adsorbent and also as a wick permitting even distribution of the solvent. When development is complete and the "sandwich" broken down, the blotting paper is gently removed. If two dimensional chromatograms are being done, a new series of blotting paper strips are employed for the 2nd solvent.

We have employed this technique in the separation of amino acids and have noted no undesirable effects from the use of the blotting paper. On the contrary, we have found that, in addition to protection of the margins of the adsorbent layer, the solvent front is more even in its progress and is not delayed, but, rather, advances slightly more rapidly.

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Einfluss der Schichtqualität auf die dünnschichtchromatographische Trennung von Aminosäuren

Zur Dünnschichtchromatographie (DC) von Aminosäuren eignet sich Aluminiumoxid[1], Kieselgel[2–11], Kieselgel/Kieselgur[12], Cellulose[13–16] und DEAE-Cellulose[17] als Träger.

Kieselgel-Schichten bewährten sich zum Nachweis der Aminosäuren in Proteinhydrolysaten[5,6], im Urin[8,9], im Blut[7,18], im Gifte der Gelbbauchunke[10] und im Maisquellwasser[11].

Die meisten Autoren verwendeten Kieselgel-G-(Merck)-Schichten[2–10]; Kieselgel (Woelm)[9] (ohne Bindemittel) und Supergel (AGFA-Wolfen)[11] wurden gelegentlich auch benutzt. Heute stehen zur DC zahlreiche Kieselgel-Sorten zur Verfügung; in der vorliegenden Arbeit soll ihre Eignung zur Trennung von Aminosäuren untersucht werden.

BRENNER *et al.*[19,20] sowie LISBOA UND DICZFALUSY[21] haben darauf hingewiesen, dass die Qualität des Sorptionsmittels die R_F-Werte z.T. erheblich beeinflusst. Wir haben einerseits Kieselgele verschiedener Provenienz und andererseits verschiedene "Chargen" ein und derselben Kieselgel-Sorte untersucht.

Experimentelles

Die Herstellung der Sorptionsschichten erfolgte nach den Angaben von BRENNER *et al.*[22] unter Anwendung des Streichgerätes von STAHL. Das Verhältnis Sorptionsmittel/Wasser betrug für eine Schichtdicke von 0.25 mm:

1:2 bei Kieselgel-G (Merck A.G., Darmstadt, Deutschland);
1:2 bei Kieselgel-H (Merck A.G., Darmstadt, Deutschland);
1:2 bei MN-Kieselgel-G-HR (Macherey & Nagel A.G., Düren, Deutschland);
6:9 bei Kieselgel-Woelm (Woelm A.G., Eschwege, Deutschland);
2:5 bei Kieselgel-D5-Camag (Camag A.G., Muttenz B.L., Schweiz).

Die Platten wurden über Nacht an der Luft getrocknet[22]. Die Chromatographie erfolgte bei Zimmertemperatur in der "grossen" Trennkammer der Fa. Desaga*, welche zur Sättigung mit Filterpapier ausgekleidet wurde. Vor Einstellung der Platten wurden die Kammer kräftig geschüttelt[22].

Als Fliessmittelkomponente verwendeten wir Lösungsmittel zur Chromatographie oder p.a. Qualität.

Sämtliche Chromatogramme wurden zur gleichen Zeit ausgeführt. Das Volumen des Fliessmittels betrug jeweils 150 ml.

Aufgetragen wurden je 1 μg Aminosäure in 1 μl 0.1 N HCl. Zur Revelation benutzten wir Ninhydrin[4]. Bei schwertrennbaren Substanzen bewährte sich die Arbeitsweise von BRENNER UND NIEDERWIESER[4] und die kalte Ninhydrin-Reaktion nach OPIENSKA-BLAUTH *et al.*[23].

Ergebnisse

Die Figs. 1–5 zeigen die zweidimensionale Trennung der wichtigsten Aminosäuren unter identischen Versuchsbedingungen. Die Trennleistung der einzelnen Kiesel-

* Desega, G.m.b.H., Heidelberg (Deutschland).

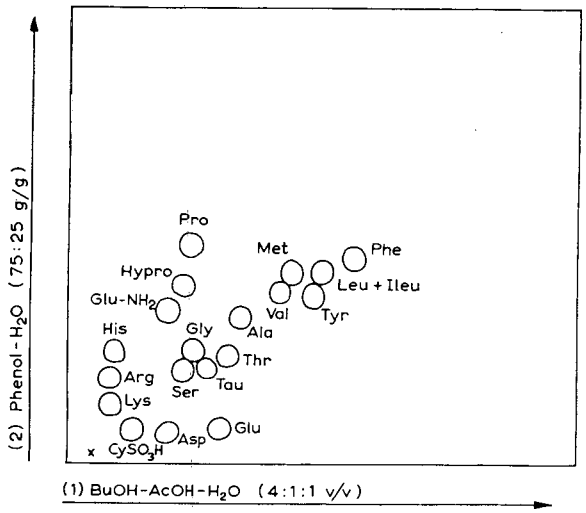

Fig. 1 Zweidimensionale Trennung der Aminosäuren auf Kieselgel-G-Schichten.

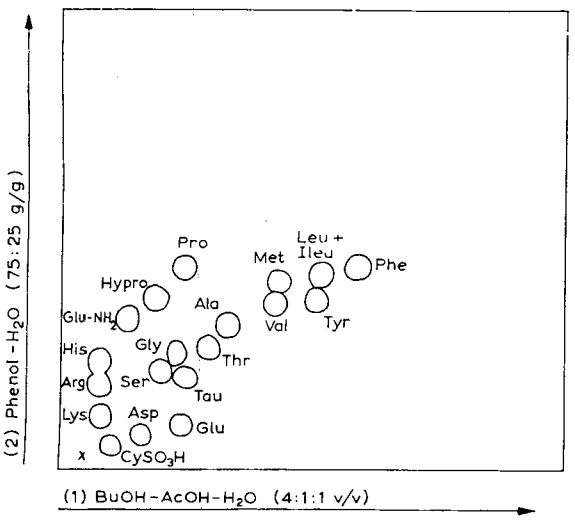

Fig. 2. Zweidimensionale Trennung der Aminosäuren auf MN-Kieselgel-G-HR-Schichten.

gele lässt sich beurteilen, wenn man die Trennung der Komponente einiger Verbindungsgruppen miteinander vergleicht*.

Basische Aminosäuren. Die Trennung von *Lysin*, *Arginin* und *Histidin* gelingt am besten mit Kieselgel-G. Auf Kieselgel-H-Schichten und auf MN-Kieselgel-G-HR-Schichten rücken diese Verbindungen näher zueinander. Hierdurch wird jedoch die Unterscheidung kaum beeinträchtigt. Alle drei basischen Aminosäuren überlappen

* Eine Deformation der Flecken (vgl. dazu *Whatman Technical Bulletin* C3) konnte unter unseren Versuchsbedingungen nicht beobachtet werden.

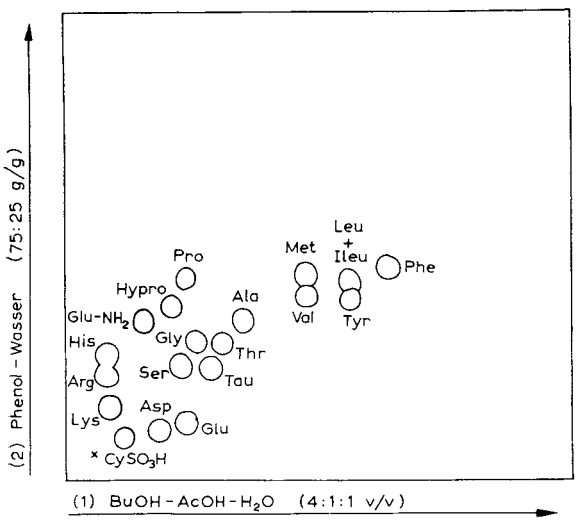

Fig. 3. Zweidimensionale Trennung der Aminosäuren auf Kieselgel-H-Schichten.

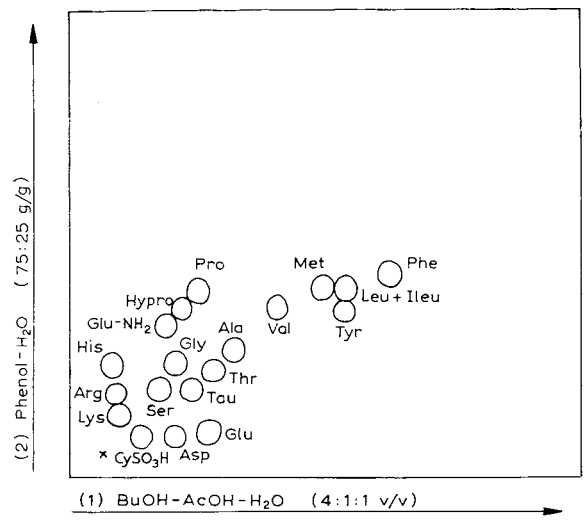

Fig. 4. Zweidimensionale Trennung der Aminosäuren auf Kieselgel-Woelm-Schichten.

teilweise auf Kieselgel-Woelm und auf Kieselgel-Camag. Sie lassen sich jedoch ohne besondere Schwierigkeit unterscheiden, wenn die Arbeitsweise von BRENNER UND NIEDERWIESER[4] oder von OPIENSKA-BLAUTH *et al.*[23] zur Revelation angewandt wird.

Serin, Glycin und Taurin. Verwendet man Kieselgel-H oder Kieselgel-Woelm-Schichten (beide ohne Bindemittel), so ist die Trennung dieser Aminosäuren vorzüglich. Die Wanderungsgeschwindigkeit von *Serin* und *Glycin* ist auf Kieselgel-G nur wenig verschieden, auch *Taurin* liegt in der unmittelbaren Nähe. Auf Kieselgel-Camag und auf MN-Kieselgel-G-HR überlappen *Serin, Glycin* und *Taurin* bzw. *Serin* und *Glycin* ohne dass die Unterscheidung dadurch verunmöglicht würde.

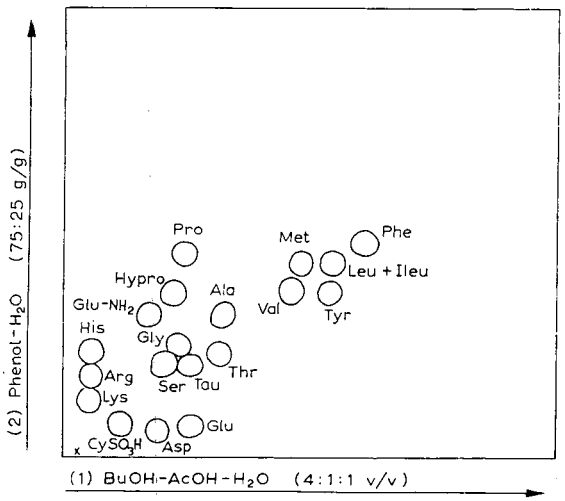

Fig. 5. Zweidimensionale Trennung der Aminosäuren auf Kieselgel-Camag-D5-Schichten.

Schnellwandernde Aminosäuren. Valin, Methionin, Tyrosin, Leucin (Isoleucin) und *Phenylalanin* lassen sich mit Kieselgel-Camag bzw. Kieselgel-G als stationäre Phase vorzüglich bzw. befriedigend trennen. Ihre Unterscheidung ist zwar möglich, jedoch die Trennung unvollständig auf MN-Kieselgel-G-HR und auf Kieselgel-Woelm (Met + Val und Tyr + Leu bzw. Met + Leu + Tyr). Zur Trennung von *Leucin* und *Isoleucin* ist die Durchlaufchromatographie erforderlich (FAHMY *et al.*[5]).

Glutamin, Hydroxyprolin und Prolin. Die Trennung dieser Gruppe bereitet allgemein keine Schwierigkeiten; die Auflösung ist jedoch auf Kieselgel-Woelm-Schichten nur unvollständig.

Nimmt man an, dass die Trennung der Aminosäuren von Qualitätsschwankungen in der Fabrikation bei ein und derselben Kieselgel-Sorte unabhängig ist, so kann man auf Grund der bisherigen Betrachtungen für jedes Trennproblem die geeignete stationäre Phase aussuchen. Hierbei ist von besonderer Bedeutung, dass man *hochgereinigte Kieselgele* (z.B. MN-Kieselgel-G-HR) zur Trennung verwenden kann. Verwendet man nämlich zum Nachweis der Aminosäuren an Stelle von Ninhydrin

TABELLE I

VERGLEICH VERSCHIEDENER KIESELGEL-CHARGEN UNTER IDENTISCHEN VERSUCHSBEDINGUNGEN

+++ = gute Trennung; ++ = befriedigende Trennung; + = teilweise Trennung.

Charge	*Basische Aminosäuren*	*Serin, Glycin und Taurin*	*Schnellwandernde Aminosäuren*	*Glutamin, Hydroxyprolin und Prolin*
I	+++	+*	++	+++
II	++	+*	++	+++
III	+–	+*	++	+++

* Die Unterscheidung gelingt mit der kalten Ninhydrin-Reaktion oder beim vorsichtigen Erwärmen und sofortiger Markierung der Flecken.

andere Reagenzien, welche die Aminosäuren nicht zerstören, so kann man nach Elution der Substanzen ihre Identität durch Rechromatographie sicherstellen. Ein solches Vorgehen ist insbesondere bei der Untersuchung vom komplexen biologischen Material bedeutungsvoll. Auch die quantitative Bestimmung wird bei dieser Arbeitsweise erleichtert, weil keine, oder nur wenige, Verunreinigungen eine U.V.-Bestimmung stören. Geeignete Reagenzien sind z.B. 2,4-Dinitrofluorbenzol oder Phenylisothiocyanat. Auf die zerstörungsfreie Revelation von Aminosäuren auf Dünnschichtchromatogrammen werden wir einer folgenden Arbeit zurückkommen.

Vorläufige Versuche haben ergeben, dass die Trennung der Aminosäuren auf verschiedenen "Chargen" von Kieselgel-G nur wenig verschieden ist. Obschon die R_F Werte bei den untersuchten drei "Chargen" voneinander abweichen, ist lediglich die Trenngüte von Lysin und Arginin geringfügig unterschiedlich (Tabelle I).

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Dünnschichtchromatographie von Chinonen, Hydroxychinonen und Phenolen an acetyliertem Polyamid

Polyamide vom Typ des Perlons (Polycaprolactam) und Nylons (Hexamethylendiamin-adipat) eignen sich ausgezeichnet als Säulenmaterial für die Trennung von phenolischen Substanzgemischen (vgl. Literaturzusammenfassung von ENDRES UND HÖRMANN[1]). Der Trenneffekt beruht auf der Ausbildung von Wasserstoffbrücken zwischen den phenolischen Hydroxylgruppen der Phenole und den Amidbindungen des Polyamids. Die Affinität eines Phenols zum Polyamid ist umso grösser, je grösser die Anzahl der aromatischen Hydroxylgruppen ist, sofern sich die Hydroxylgruppen nicht durch intramolekulare Wasserstoffbrücken gegenseitig beeinträchtigen[2].

Chinone, die über ein additionsfähiges Kohlenstoffatom verfügen, wie *p*-Benzochinon, Methylchinon, u.a., werden an die freien Aminogruppen des Polyamids irreversibel gebunden[3,4]. Durch Acetylierung dieser freien Aminogruppen[5] wird die Addition des Chinons an das Polyamid unterbunden. Das acetylierte Polyamidpulver stellt ein gutes Säulenfüllmaterial für die Auftrennung von Chinon-, Hydroxychinon- und Phenolgemischen dar[4].

Die Anwendung von Polyamiden in der Dünnschichtchromatographie wurde von HÖRHAMMER und Mitarb.[6] zur Trennung von phenolischen Naturstoffgemischen beschrieben. Aber auch bei der Dünnschicht-Polyamidchromatographie stellten wir fest, dass verschiedene Chinone z.T. irreversibel gebunden werden und an der Auftragsstelle verbleiben. Die Reaktion zwischen Chinon und Polyamid tritt während des Auftragens und bei langsam wandernden Chinonen auch während des Entwickelns ein.

Aus diesem Grunde haben wir käufliches Polyamidpulver für die Dünnschichtchromatographie* nach der Vorschrift von GRASSMANN und Mitarb.[5] acetyliert. An dem acetylierten Polyamidpulver zeigten die oben erwähnten Chinone auch bei längerem Liegen der noch nicht entwickelten Platten keine irreversibel gebundenen Anteile.

Für die Entwicklung des Polyamidplatten erwiesen sich Wasser–Methanol- und Wasser–Aceton-Gemische als gut geeignete Lösungsmittel. Die Tabelle I gibt die R_F-Werte verschiedener Phenole, Chinone und Hydroxychinone in den Lösungsmitteln Methanol–Wasser (1:1) und Aceton–Wasser (3:1) wieder. Es ist jedoch zu beachten, das selbst unter Einhaltung konstanter Temperatur und bei Chromatographie mit der gleichen Charge von Polyamidpulver beträchtliche Abweichungen bei den einzelnen R_F-Werten auftreten. Dies könnte auf der ungleichmässigen Dicke der Polyamidschichten beruhen. Die in Tabelle I angegebenen R_F-Werte sind Mittelwerte mehrerer Versuche, wobei jeweils sechs Substanzen auf einer Platte aufgetragen wurden.

Die in Tabelle I angegebenen R_F-Werte entsprechen nicht denen die bei entsprechenden Substanzen an der Polyamidsäule gefunden wurden. So ist nicht nur die hohe Affinität verschiedener Chinone (vgl. Naphtochinon-1,4) zum Polyamidpulver der Dünnschichtplatten überraschend, sondern besonders das Verhalten der drei Dihydroxybenzole, wie die Tabelle II veranschaulicht, in der die R_F-Werte von

* Polyamidpulver für die Dünnschichtchromatographie der Firma Merck, Darmstadt und der Firma Woelm, Eschwege.

TABELLE I

R_F-WERTE VERSCHIEDENER PHENOLE UND CHINONE AN ACETYLIERTEM POLYAMIDPULVER BEI DER DÜNNSCHICHTCHROMATOGRAPHIE

Substanz	R_F-*Werte*	
	Methanol–Wasser (1:1)	*Aceton–Wasser* (3:1)
Brenzcatechin	0.36	0.59
Resorcin	0.33	0.76
Hydrochinon	0.46	0.65
Protocatechusäure	0.34	0.57
Gallussäure	0.30	0.66
Pyrogallol	0.54	0.66
dl-Catechin	0.31	0.64
Benzochinon-1,4	0.72	0.86
2-Methylchinon-1,4	0.39	0.74
2,5-Dimethylchinon-1,4	0.37	0.65
Naphtochinon-1,4	0.11	0.61
3-Hydroxynaphtochinon	0.10	0.44
Vitamin K_3	0.05	0.49
Ubichinon	0.00	0.00

Brenzcatechin, Resorcin und Hydrochinon an einer Polyamidsäule angegeben sind. Brenzcatechin hat an der Säule in allen untersuchten Lösungsmittelsystemen eine geringere Affinität zum Polyamid und damit einen höheren R_F-Wert als Resorcin und Hydrochinon. Bei der Dünnschichtchromatographie trifft dies aber nicht mehr zu (vgl. Tabelle I).

TABELLE II

R_F-WERTE VERSCHIEDENER DIHYDROXYBENZOLE AN EINER POLYAMIDSÄULE[1]

Substanz	R_F-*Werte*	
	Aceton–Wasser (1:4)	*Äthanol–Wasser* (1:1)
Brenzcatechin	0.46	0.62
Resorcin	0.40	0.51
Hydrochinon	0.39	0.55

Wir haben die stärkere Affinität des Resorcins und Hydrochinons zum Polyamid damit erklärt, dass bei diesen Phenolen beide Hydroxylgruppen mit verschiedenen Peptidbindungen des Polyamids in Wechselwirkung treten können. Die Hydroxylgruppen des Brenzcatechins müssen dagegen um die gleiche Peptidgruppe konkurrieren. Zudem muss beim Brenzcatechin eine teilweise Absättigung durch intramolekulare Wasserstoffbrücken in Betracht gezogen werden, die ebenfalls die Affinität zum Polyamid herabsetzen[1,2].

Dieses unterschiedliche Verhalten bei Säulen- und Dünnschichtchromatographie kann nicht an der Acetylierung des Polyamids liegen, da die R_F-Werte an

einer Polyamidsäule und an einer Säule mit acetyliertem Polyamid keine grossen Unterschiede zeigen[4]. Auch auf Polyamidplatten, also auf Material, welches nicht acetyliert wurde, zeigt Brenzcatechin einen geringeren, oder nahezu den gleichen R_F-Wert wie Resorcin und Hydrochinon, wie Tabelle III veranschaulicht.

TABELLE III

R_F-WERTE VERSCHIEDENER DIHYDROXYBENZOLE AN POLYAMID-DÜNNSCHICHTPLATTEN

Substanz	*R_F-Werte*		
	Methanol–Wasser (1:1)	*Aceton–Wasser (3:1)*	*Aceton–Wasser (1:4)*
Brenzcatechin	0.25	0.65	0.23
Resorcin	0.33	0.67	0.18
Hydrochinon	0.39	0.70	0.24

Ein möglicher Grund für dieses unterschiedliche Verhalten der einfachen Phenole und der Chinone in der Polyamid-Dünnschicht- und Polyamid-Säulenchromatographie könnte darin zu sehen sein, dass es sich bei der Säulenchromatographie um in Wasser gequollenes Polyamid (vgl. hierzu GRASSMAN und Mitarb.[7]), bei der Dünnschichtchromatographie jedoch um ungequollenes Polyamid handelt.

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Polyamid-Dünnschichtchromatographie vegetabilischer Gerbstoffextrakte

Die Papierchromatographie handelsüblicher vegetabilischer Gerbstoffextrakte, die komplizierte Mischungen sehr ähnlich aufgebauter Polyhydroxyphenole verschiedener Molekulargrössen darstellen, ergibt keine befriedigende Auftrennung. Besonders störend machen sich dabei die braunen Kondensationsprodukte der Gerbstoffe bemerkbar, die in verschiedenen Lösungsmitteln über die gesamte Laufläche verschmiert werden.

Nachdem sich Polyamidpulver als Säulenfüllmaterial für die Auftrennung der Fichtenbast-Gerbstoffvorstufen[1] und später auch für weitere Gerbstoffe und viele andere phenolische Naturstoffgemische (vgl. zusammenfassende Arbeit von ENDRES UND HÖRMANN[2]) sehr gut bewährt hat, haben wir in der vorliegenden Arbeit verschiedene vegetabilische Gerbextrakte an Polyamid-Dünnschichtplatten untersucht.

Polyamidpulver als Trägersubstanz für Dünnschichtchromatographie, erstmals von HÖRHAMMER, WAGNER UND LEEB[3] angewendet, hat hier gegenüber anderen, für die Dünnschichtchromatographie bekannten Trägersubstanzen den Vorteil, dass die kondensierten Gerbstoffe mit ihrer starken Affinität zum Polyamid praktisch an der Auftragsstelle sitzen bleiben. Wir haben aus diesem Grund handelsübliche Gerbstoffextrakte, die besonders reich an Kondensationsprodukten sind, verwendet. Weiter war uns daran gelegen, eine einfache und schnelle Methode zur Identifizierung solcher Extrakte zu finden.

Sehr viele Gerbextrakte, besonders die, die zur Klasse der kondensierten Gerbstoffe gehören, enthalten Verbindungen, die im U.V.-Licht eine intensive Fluoreszenz zeigen. Deshalb wurden die Platten nach der Entwicklung zuerst im U.V.-Licht betrachtet, die fluoreszierenden Flecken markiert und die Platten dann mit einer verdünnten alkoholischen Lösung von Eisen(III)chlorid besprüht.

Von den verschiedenen Lösungsmittelmischungen, die wir für die Entwicklung der Chromatogramme verwendet haben, erwiesen sich 50–75%iges Aceton und 50–75 %iges Äthanol als gut geeignet. Ein geringerer Gehalt an organischem Lösungsmittel zeigte in beiden Fällen einen schlechteren Trenneffekt. Keine guten Ergebnisse erhielten wir mit Dioxan-, Tetrahydrofuran-, Äthylmethylketon- und Dimethylformamid–Wasser-Gemischen. Am besten erwiesen sich schliesslich die in Tabelle I angeführten Lösungsmittelgemische.

TABELLE I

LÖSUNGSMITTELGEMISCHE, DIE SICH GUT FÜR DIE AUFTRENNUNG VON VEGETABILISCHEN GERBSTOFFEXTRAKTEN AN POLYAMID-DÜNNSCHICHTPLATTEN EIGNEN

Aceton–Methanol–Wasser (5:4:1)
Aceton–Methanol–1 *M* Essigsäure (2:7:1) und (5:4:1)
Aceton–Methanol–1 *M* Pyridin (2:7:1) und (5:4:1)
Aceton–Propanol–Wasser (5:4:1)
Tetrahydrofuran–Methanol–Wasser (3:5:2)
Tetrahydrofuran–Äthanol–Wasser (5:3:2) und (3:5:2)
Tetrahydrofuran–Dioxan–Wasser (3:5:2)
Aceton–Tetrahydrofuran–Methanol–Wasser (5:2:2:1)
Aceton–Dimethylformamid–Methanol–Wasser (4:2:3:1) und (2:3:4:1)

Nachfolgende Tabelle II zeigt die R_F-Werte von sechs handelsüblichen Gerbextrakten in einigen der genannten Lösungsmittelgemische.

Eine wesentlich bessere Auftrennung kann dadurch erreicht werden, dass mit demselben Lösungsmittelgemisch mehrmals in der gleichen Richtung entwickelt wird, wobei es allerdings sehr wichtig ist, dass die Lösungsmittelgemische vor jeder Chromatographie neu angesetzt werden. Desgleichen müssen die Platten vor der zweiten und jeder weiteren Chromatographie an der Luft gut getrocknet werden. Wie die Tabelle III zeigt, wird z.B. bei dreimaliger Entwicklung ein Quebrachoextrakt in neun gut abgesetzte fluoreszierende Flecken aufgetrennt, gegenüber einer Auf-

TABELLE II

R_F-WERTE VERSCHIEDENER HANDELSÜBLICHER GERBEXTRAKTE AN POLYAMID-DÜNNSCHICHTPLATTEN

Lösungsmittel: I = Aceton–Methanol–1 *M* Pyridin (5:4:1)
II = Aceton–Propanol–Wasser (5:4:1)
III = Aceton–Methanol–Wasser (5:4:1)

*R_F-Werte und Farbe der U.V.-Fluoreszenz (in Klammern)**

Mimosa			*Fichte*			*Quebracho*			*Myrobalanen*			*Eiche*			*Sumach*		
I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
0.71 (ge)	0.75 (gr)	0.71 (ge)	0.76 (bl)	0.76 (bl)	0.73 (bl)	0.68 (ge)	0.68 (ge)	0.61 (ge)	0.55 (br)	0.23 (gr)	0.73 (br)	0.81 (ga)	0.08 (gr)	0.76 (br)	0.49 (br)	0.49 (br)	0.05 (br)
0.61 (ge)	0.64 (gr)	0.61 (ge)	0.49 (bl)	0.54 (ge)	0.50 (bl)	0.59 (ge)	0.61 (ge)	0.56 (ge)	0.25 (ge-gr)	0.10 (gr)	0.37 (ge)	0.65 (ga)		0.63 (br)	0.25 (ge)	0.25 (br)	0.25 (ge)
0.46 (ge)	0.50 (ge)	0.47 (ge)	0.35 (ge)	0.49 (bl)	0.38 (ge)	0.46 (ge)	0.51 (ge)	0.50 (ge)	0.15 (ge-gr)		0.29 (ge)						
0.29 (ge)	0.28 (ge)	0.32 (ge)	0.25 (ge)	0.30 (ge)	0.28 (ge)	0.34 (ge)	0.41 (ge)	0.43 (ge)	0.10 (gr)		0.07 (gr)	0.43 (br)		0.08 (gr)	0.08 (br)	0.03 (ge)	
0.23 (ge)		0.27 (ge)				0.27 (ge)	0.33 (ge)					0.11 (gr)					

* bl = blau; ga = grau; ge = gelb; br = braun; gr = grün.

trennung in nur fünf Flecken bei einmaliger Chromatographie. Ähnliches gilt auch für die anderen Gerbextrakte (Tabelle III).

Infolge der zum Beschicken der Glasplatten erforderlichen Dünnflüssigkeit der Polyamidpulver-Aufschlämmung ist es sehr schwer eine bestimmte gleichmässige Schichtdicke zu erreichen. Das führt trotz Einhaltung gleicher Versuchsbedingungen gelegentlich zu unterschiedlichen R_F-Werten (vgl. hierzu GRAU UND ENDRES[4]).

TABELLE III

R_F-WERTE VERSCHIEDENER GERBEXTRAKTE AN POLYAMID-DÜNNSCHICHTPLATTEN

Lösungsmittel: Aceton–Methanol–1 *M* Pyridin (5:4:1).
a = einmalige, b = dreimalige Chromatographie.

*R_F-Wert und Farbe der U.V.-Fluoreszenz (in Klammern)**											
Mimosa		*Fichte*		*Quebracho*		*Myrobalanen*		*Eiche*		*Sumach*	
a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.71	0.81 (br)	0.76	0.87 (bl)	0.68	0.88 (br)	0.55	0.65 (gr)	0.81	0.80 (gr)	0.49	0.69 (br)
0.61	0.69 (ge)	0.49	0.81 (bl)	0.59	0.80 (ge)	0.25	0.35 (gr)	0.65	0.63 (ga)	0.25	0.32 (ga-br)
0.46	0.61 (ge)	0.35	0.70 (gr)	0.46	0.72 (ge)	0.15	0.21 (gr)	0.43	0.17 (ga)	0.08	0.12 (ge)
0.29	0.38 (ge)	0.25	0.63 (gr)	0.34	0.65 (ge)	0.10	0.11 (gr)	0.11	0.12 (gr)		0.06 (br)
0.23	0.33 (ge)		0.53 (ge-br)	0.27	0.48 (ge)		0.06 (gr)		0.05 (gr)		
	0.05 (ge)		0.39 (ge)		0.43 (ge)						
			0.32 (ge)		0.37 (ge)						
			0.12 (ge)		0.10 (ge)						
			0.05 (ge)		0.06 (ge)						

* bl = blau; br = braun; ga = grau; ge = gelb; gr = grün.

Nach einem leichten Besprühen mit Fixierlack (Fa. C. Roth, Karlsruhe) lässt sich die Polyamidschicht mittels einer (Mipo)folie ablösen und nach ebenfalls leichtem Besprühen auf der Rückseite und Verwendung einer zweiten Folie als Original gut aufbewahren*.

Durchführung der Arbeit

Zum Auftragen des Polyamids auf die Glasplatten wird dieses nur in Methanol aufgeschlämmt und die Platten mit den üblichen Auftraggeräten beschickt. Es wurden Polyamidpräparate für Dünnschichtchromatographie der Firmen Merck, Darmstadt und Woelm, Eschwege verwendet. Vergleichsversuche zeigten, dass beide Präparate zu ähnlichen Ergebnissen führen, doch liessen sich einige unserer Substanzgemische mit dem Polyamidpulver von Woelm etwas besser auftrennen.

Der Laufweg der verwendeten Lösungsmittelgemische betrug durchwegs

* Frau CH. SCHIPPEL danken wir für die Durchführung der Versuche.

15 cm von der Auftragsstelle. Bei mehrmaligem Entwickeln der gleichen Platte in gleicher Richtung stieg das Lösungsmittel im ersten Lauf 5 cm, im zweiten 10 cm und erst im dritten 15 cm. Bei diesen Versuchen wurden die Lösungsmittel jedesmal neu angesetzt.

Die zu untersuchenden Gerbstoffextrakte wurden entweder in 50%igem Aceton oder in Methanol gelöst und vom Rückstand abdekantiert. Die Methanollösung unterschied sich im chromatographischen Bild nicht von der Acetonlösung.

Die Dünnschichtplatten wurden im langwelligen U.V.-Licht (365 mμ) ausgewertet, die Ergebnisse durch Farbaufnahmen festgehalten. Dafür wurde der Agfa C18 Film verwendet mit einer Sperrfilterkombination 2mm GG4 und 2mm GG13 von Schott. Bei Blende 8 bringt eine Belichtungszeit von 15 Sek. bei Verwendung von zwei Hanau-U.V.-Lampen (PL 327) in einem Abstand von etwa 40 cm die beste Wiedergabe.

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Thin-layer chromatography of tetracyclic triterpenes on silica impregnated with silver nitrate

The rapid, reversible coordination of silver ions with unsaturated compounds, studied extensively by LUCAS and coworkers[1,2], can be expected (NICHOLS[3]) to be useful in the separation of saturated and unsaturated compounds, such as fatty acid esters. Indeed, this principle has been applied by DUTTON, SCHOLFIELD AND JONES[4] to mixtures of isomeric fatty acid esters, by DE VRIES[5] to lipids, by BARRETT, DALLAS AND PADLEY[6] to glycerides, by MORRIS[7] to cholesteryl esters (see also AVIGAN, DE

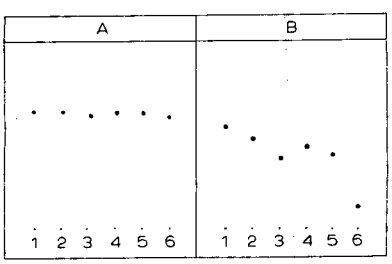
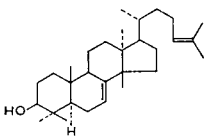
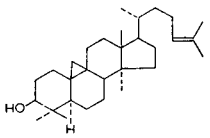


Fig. 1. (A) Silica gel G. (B) Silica gel G, impregnated with silver nitrate (5% w/w).


(I)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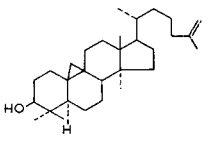
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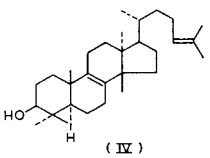
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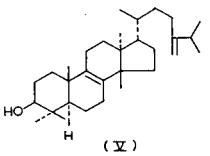
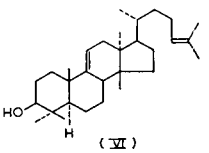

(V)


(VI)

I Butyrospermol
II Cycloartenol
III Cyclolaudenol
IV Euphol
V α-Euphorbol
VI Parkeol

GOODMAN AND STEINBERG[8]) and to fatty acid methyl esters[9], and by SHABTAI, HERLING AND GIL-AV[10] to cyclic olefins. Recently, thin layers impregnated with silver nitrate have also been used for the separation of sesquiterpene hydrocarbons[11].

In the present study, this method has been extended to include some tetracyclic triterpenes. The compounds studied had been shown to have practically the same R_F values on thin layers of alumina G[12]. However, on silica gel G, impregnated with silver nitrate, the R_F values were sufficiently different to enable the substances to be identified separately (Fig. 1). The values and the colors obtained by spraying the spots with three reagents are summarized in Table I.

TABLE I

Triterpene	*R_F on silica gel G*		*Colors with spray reagent**					
			A		*B*		*C*	
	Treated with $AgNO_3$	*Untreated*	*Room temp.*	*After charring*	*Room temp.*	*After charring*	*Room temp.*	*After charring*
Butyrospermol (I)	0.40	0.42	—	brown	light yellow	blue green	light orange	brown
Cycloartenol (II)	0.33	0.42	—	brown	orange	blue green	orange	brown
Cyclolaudenol (III)	0.26	0.41	—	brown	light yellow	light green	light orange	dark brown
Euphol (IV)	0.30	0.42	—	brown	light yellow	light green	yellow	brown
α-Euphorbol (V)	0.27	0.42	—	brown	light yellow	light green	yellow	brown
Parkeol (VI)	0.11	0.41	—	brown	light pink	light green	light yellow	light brown

* Spray reagents: A = 50% sulfuric acid in water[13]; B = 10% phosphomolybdic acid in ethanol[14]; C = a mixture of chlorosulfonic and acetic acids (1:2)[11].

For convenience the structural formulae of the triterpenes are drawn.

Although the number of examples is too small to draw significant conclusions as to the correlation between R_F and structure, it is interesting to note the differences in the values for butyrospermol (I), euphol (IV) and parkeol (VI), which differ from each other in the position of the ring double bond. Even the shift of the double bond of the side chain, in passing from cycloartenol (II) to cyclolaudenol (III), has a distinct influence on the migratory tendency.

Experimental

Preparation of plates. The suspension for five plates (20 × 20 cm) was prepared by shaking 30 g of silica gel G and 60 ml of water for 30 sec and applied uniformly to a thickness of 0.25 mm with a Desaga applicator. After 30 min at room temperature, the plates were heated in an oven at 125–130° for 45 min. After cooling they were sprayed with concentrated aqueous-methanolic silver nitrate solution, 5 % relative to silica gel, and then activated at 120° for 30 min.

This method permits impregnation of only part of the plate, which can thus be used for comparative chromatography.

The plates were used immediately after cooling.

Development. The samples were dissolved in chloroform (1 mg/1 ml), and applied with micropipettes along a line 2 cm above the edge of the plate. The experiments were performed at room temperature (24–27°). Chloroform was used as mobile phase; it was allowed to ascend to a distance of 15 cm. The plates were removed and dried in air.

Detection. The triterpenes were detected by spraying with the three reagents, followed by heating in an oven at 150° for 15 min. The colors of the spots were recorded before and after charring.

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Quantitative thin-layer chromatography on aluminium foil

Since the introduction of thin-layer chromatography, efforts have been made to find methods for the quantitative evaluation of the separated substances. Among these methods the most reliable seem to be those in which extraction of the substance from the adsorbent is followed by quantitative analysis of the extract. Quantitative removal of the relevant part of the adsorbent (the "spot") from the glass plate is necessary. This is obviously a drawback of the thin-layer technique in comparison with paper chromatography, where the spot is simply cut out. After removal of the adsorbent some substance may adhere to the glass plate and it is not surprising that low recoveries often occur. Simultaneous analysis of known amounts of the substance as a reference standard is of course advisable[1].

The present method obviates the necessity of removing the adsorbent from the glass plate and reverts to cutting the spot out of the layer. On the other hand, it profits fully by the perfect flatness of a glass plate, which makes it possible to obtain a uniform layer of adsorbent.

For this purpose aluminium foil is fixed to a glass plate and the thin layer of adsorbent is spread on the aluminium. After chromatography the spots are cut out.

Experimental

Materials

Glass plate: *e.g.* 1500 × 500 × 5 mm, smooth surface
Aluminium foil: *e.g.* Indufol, 1000 × 300 mm
Strips of filter-paper: 1200 × 70 mm
Scotch tape: 50 mm
Stainless-steel frame: 200 × 200 × 30 mm
Hydrofluoric acid: 1 % aqueous solution in water

Method

Paste with water 100 cm aluminium foil on the plate and make the surface flat, using a piece of cloth. Degrease the aluminium foil with hydrofluoric acid. Rinse with with water and dry. Fix two strips of filter-paper on the foil by means of Scotch tape, leaving a strip of approximately 18.5 cm of the foil visible (see Fig. 1). Pour the slurry of the adsorbent on the foil and spread it with a glass rod[2]. Allow to stand for half an hour in the open air, then remove the filter-paper. Cut the foil into squares of 18 × 18

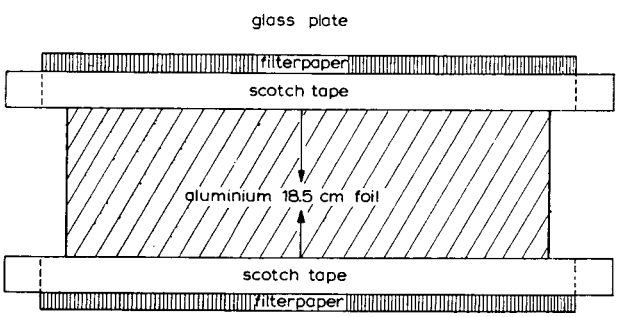

Fig. 1. Aluminium foil on a glass plate, ready for covering.

cm with a lancet and mount these on frames. Dry at 110° for one hour and the framed thin layer is ready for use.

The thickness of the layer (0.2 mm) may be varied by using more strips of filter paper. The separation on aluminium foil is the same as on a glass plate.

Partition chromatography of 2,4,5,4'-tetrachlorodiphenyl sulphide

For this purpose thin layers were prepared consisting of silica gel with polyethylene glycol as a stationary phase[3]. The slurry for coating was obtained by stirring 30 g of silica gel GF 254 (purified by extraction with methanol and methylene chloride) with a solution of 3 ml of polyethylene glycol in 60 ml of distilled water.

A micro pipette was used to deliver 200 μl of petroleum ether, containing 100 to 800 μg of the sulphide, on the silica gel layer. The chromatogram was developed with *n*-hexane using an ascending technique until the solvent front had ascended a distance of 17 cm. The foil was dried in the open air for 3 minutes and rechromatographed twice in the same way.

TABLE I

RECOVERY OF 2,4,5,4'-TETRACHLORODIPHENYL SULPHIDE

	Without running	*After 3 runs*
Average recovery	99.5%	100.1%
No. of determinations	5	8
Range	98.3–100.1%	99.9–100.5%
Standard deviation	0.73	0.25
R_F after 1 run	—	0.3

After the third run the foil was dried for 3 minutes. The location of the spot was clearly visible in ultraviolet light (254 nm); in this light the dark spot was cut out and the snip extracted with methanol. The absorption was measured spectrophotometrically at 258 nm ($a^{1\%}_{1cm} = 500$).

The results are given in Table I.

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Séparation du cholestérol, du desmostérol et du 5-dihydrocholestérol par chromatographie en couche mince après propionylation

La séparation par chromatographie en couche mince de certains stérols comme le cholestérol, le desmostérol (24-deshydrocholestérol) et le 5-dihydrocholestérol s'avère assez difficile.

Actuellement, la méthode la plus valable est celle qui a été proposée par AVIGAN *et al.*[1] et qui consiste, après acétylation préalable du mélange de stérols selon la technique de JOHNSTON *et al.*[2] à réaliser une migration sur plaque de 40 × 20 cm imprégnée d'acide silicique, sous l'action d'un solvant hexane–benzène (5:1, v/v).

Ce procédé malgré son bon pouvoir de résolution présente au moins deux inconvénients:

(1) Il s'avère particulièrement long à mettre en oeuvre.

(2) Il nécessite un matérial spécial (plaques de 40 × 20 cm) car dans les conditions classiques (plaques 20 × 20 cm) la séparation est médiocre du fait des faibles différences de R_F.

Dans le présent travail, une méthode de séparation efficace et rapide, sur plaques de 20 × 20 cm est décrite, faisant appel à une propionylation préalable des stérols et à une migration sur couche de silicagel imprégné de nitrate d'argent.

Méthode expérimentale

(1) *Préparation de l'ester propionique.* Le stérol ou l'extractum renfermant les stérols est directement mis en contact avec 0.5 ml de chlorure de propionyle*. On facilite la dissolution par léger chauffage à la flamme d'un bec bunsen et laisse ensuite refroidir 10 min. On ajoute alors 5 ml d'hexane, agite, et 3 lavages successifs de la phase organique sont alors pratiqués à l'aide d'eau distillée, de carbonate acide de potassium en solution aqueuse à 10 % et finalement d'eau distillée. La couche organique est alors décantée en tube à essai jaugé à 5 ml, complétée à ce volume par de l'hexane. Le dosage des stérols peut être réalisé sur une partie aliquote de cette solution. Le volume résiduel est évaporé sous vide.

(2) *Préparation des plaques.* 8 g de nitrate d'argent sont dissous extemporanément dans 60 ml d'eau distillée. La mise en suspension dans ce volume de 30 g de silicagel** est effectuée très rapidement et le mélange est étalé*** sur 3 plaques de 20 × 20 cm en réglant l'épaisseur de la couche à 450–500 μ. Elles sont mises à sécher alors 30 min à l'obscurité sous hotte ventilée, puis 2–3 h en étuve à 50°. Les plaques peuvent être conservées 3–4 jours à l'obscurité.

La viscosité de la suspension de silicagel dans une solution de nitrate d'argent est supérieure à celle d'une suspension dans l'eau distillée. De plus, des couches de 350–400 μ s'avèrent plus homogènes après dessication. Ce sont les raisons qui obligent à utiliser une plus grande épaisseur d'étalement qu'il n'est habituel, tous les étaleurs commerciaux étant étalonnes en fonction de la viscosité du Kieselgel.

(3) *Développement chromatographique.* 100–200 μg de stérols propionylés sont appliqués en solution chloroformique sur une plaque imprégnée de nitrate d'argent. Cette plaque est mise à migrer à l'obscurité dans un solvant hexane–benzène (5:1, v/v)

* Produits RP, Prolabo, Paris.
** Kieselgel G., Merck, Darmstadt.
*** Etaleur automatique Stratomat, Chemetron, Milan.

jusqu'à ce que celui-ci ait parcouru toute la plaque. La cuve à chromatographie est alors ouverte de 15 mm et on abandonne ainsi 45 min à 20°. L'évaporation de la partie supérieure permet l'ascension continue du solvant et l'entraînement des corps en cours de migration.

(*4*) *Séchage et révélation*. On sèche 30 min à 50° puis quelques heures à la température ordinaire à l'obscurité. La révélation est faite à l'acide sulfurique à 50%, suivi d'un chauffage de 30 min à 110°. On observe des spots colorés en brun foncé.

Résultats

En utilisant ces conditions opératoires on sépare de façon très satisfaisante cholestérol, desmostérol, 7-deshydrocholestérol, 5-dihydrocholestérol comme le montre la Fig. 1. De plus, la résolution est supérieure à celle obtenue avec les dérivés acétylés des mêmes stérols comme on peut l'observer sur la Fig. 2.

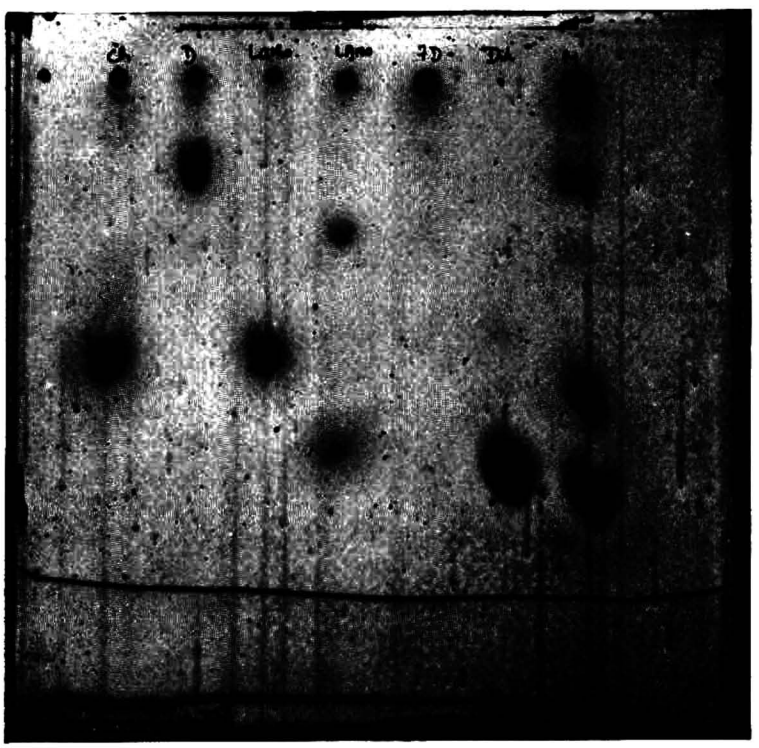

Fig. 1. Séparation des stérols propionylés sur plaque silicagel-nitrate d'argent. Solvant: hexane–benzène (5:1); révélateur: H_2SO_4 à 50%. De gauche à droite: cholestérol; desmostérol; lathostérol; lanostérol; 7-deshydrocholestérol; 5-dihydrocholestérol; mélange 7-deshydrocholestérol–desmostérol-cholestérol–5-dihydrocholestérol.

Discussion

1. Le remplacement de l'acétylation en milieu pyridiné comme l'ont proposé JOHNSTON *et al.*[2], par une propionylation par le chlorure de propionyle présente au moins deux avantages.

(a) Elle est immédiate, à l'inverse de l'acétylation pyridinée qui nécessite 12 h environ de contact.

(b) L'estérification par un acide à 3 atomes de carbone permet une migration plus rapide et une meilleure séparation dans le système solvant hexane–benzène (5:1), que l'estérification par l'acide acétique.

2. Les plaques contenant du nitrate d'argent sont préparées, non plus par pulvérisation d'une solution alcoolique de nitrate d'argent comme le préconisent AVIGAN *et al.*, mais par incorporation directe du sel d'argent dans la suspension destinée à être étalée, comme l'ont montré BARRETT *et al.*[3], HAAHTI ET NIKKARI[4], MORRIS[5], CLAUDE ET BEAUMONT[6]. Le pouvoir séparateur de la couche est ainsi bien supérieur. La proportion de nitrate d'argent incorporée n'est pas absolument critique, mais la quantité indiquée ici paraît optimale.

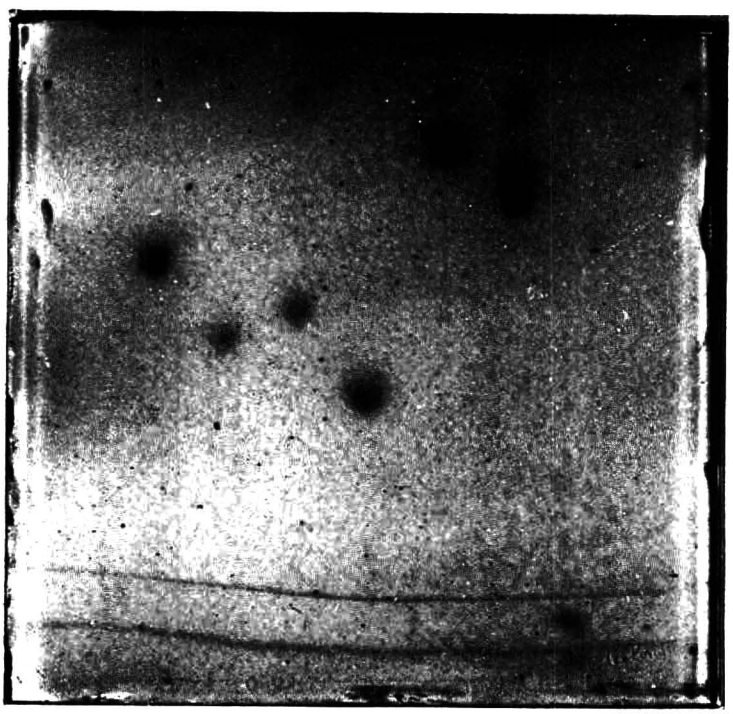

Fig. 2. Comparaison des valeurs réciproques de l'acétylation et de la propionylation pour la séparation du cholestérol, du desmostérol et du 5-dihydrocholestérol sur plaque silicagel–nitrate d'argent. Solvant: hexane–benzène (5:1); révélateur: H_2SO_4 à 50%. De gauche à droite: cholestérol acétylé; cholestérol propionylé; 5-dihydrocholestérol acétylé; 5-dihydrocholestérol propionylé; desmostérol acétyle; desmostérol propionylé.

3. L'ouverture de la cuve à chromatographie dans des conditions bien déterminées à la fin de la migration réalise un développement continu qui améliore grandement la migration globale des substances séparées.

Cette technique rapide a permis en particulier de mettre en évidence de petites quantités de desmostérol dans le sérum humain normal.

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R_F values of some estrogens and 3β-hydroxy-Δ^5-steroids in thin-layer chromatography without binder

Recently, much attention has been paid to the biological significance of 3β-hydroxy-Δ^5-steroids as the precursors of estrogen formation in the ovary and placenta. Many attempts have been made to separate estrogens, Δ^5-androstene and Δ^5-pregnene derivatives. The use of thin-layer chromatography on silica gel was systematically studied for this purpose and excellent results were obtained[1,2]. In the present communication the chromatographic technique for the separation of the most important naturally occurring estrogens and 3β-hydroxy-Δ^5-steroids on a thin layer of alumina without binder[3] is described.

Alumina without binder (activity III for 3β-hydroxy-Δ^5-steroids and activity IV for estrogens, 200–250 mesh) was freely spread on a glass plate (12 × 22 cm) and a layer 10 cm wide and 0.6–0.8 mm thick was smoothed by means of a glass rod with polythene tubing sleeves as described previously[4]. Steroid samples in chloroform were spotted on the start-line and the chromatogram was developed by the ascending technique at a slope of 15° in a chromatographic tank completely saturated with the mobile phase poured into the bottom. The solvent front reached the upper end of the glass plate within 25–30 min.

3β-Hydroxy-Δ^5-steroids were detected by spraying the plate after drying with ALLEN's reagent[5] (80 ml conc. sulphuric acid and 20 ml 90% ethanol); the purple spots appeared without heating. 7-Hydroxy-Δ^5-steroids and $\Delta^{5,7}$-dienes gave an azure-blue coloration. The sensitivity of the reaction was 1–2 μg per spot. Estrogens were detected by spraying the surface of the chromatogram while still moist with ferricyanide–ferric chloride reagent[6].

TABLE I

THE R_F VALUES (× 100) OF SOME 3β-HYDROXY-Δ^5-STEROIDS IN THIN-LAYER CHROMATOGRAPHY ON ALUMINA (ACTIVITY III) WITHOUT BINDER*

Steroid	*$CHCl_3$–EtAc*		*$CHCl_3$–EtOH*	*$CHCl_3$–EtAc-EtOH*				*CH_2Cl_2–EtAc*		*C_6H_6–EtAc–EtOH*	*C_6H_6–EtOH*
	(90:10)	(93:7)	(97:3)	(87:12:1)	(89:7:4)	(90:7:2)	(92:7:1)	(80:20)	(90:10)	(86:10:4)	(96:4)
Δ^5-Androstene-3β,7α,17β-triol	2	1	27	4		9	8			4	3
Δ^5-Pregnene-3β,17α,21-triol-20-one	2	2	32	5		12	9			8	5
Δ^5-Androsten-3β-ol-7,17-dione	2	2	36	6		11	10			9	5
Δ^5-Androstene-3β,7α-diol-17-one	3	3	36	7	41	17	11	4	2	7	5
Δ^5-Pregnene-3β,17α,20α-triol	6	5	50	11		24	19			13	10
Δ^5-Androstene-3β,16α-diol-17-one	13	11	57	21	60	33	30	16	8	19	13
Δ^5-Pregnene-3β,21-diol-20-one	16	15	59	27		37	32			22	18
Δ^5-Androstene-3β,17β-diol	26	25	59	38		44	40			28	19
Δ^5-Pregnene-3β,17α-diol-20-one	29	27	64	39		45	42	44	25	30	20
Δ^5-Pregnene-3β,20α-diol	29	28	64	39	70	50	48	50	27	32	22
$\Delta^{5,7}$-Androstadien-3β-ol-17-one				40		52	49			33	24
Δ^5-Androsten-3β-ol-17-one	54	48	68	53	77	53	52	70	50	40	30
Δ^5-Pregnen-3β-ol-20-one	68	60	69	55	79	60	58	73	53	42	35

* $CHCl_3$ = chloroform (without ethanol); CH_2Cl_2 = dichloromethane; C_6H_6 = benzene; EtOH = abs. ethanol; EtAc = ethyl acetate.

TABLE II

THE R_F VALUES (× 100) OF SOME ESTROGENS IN THIN-LAYER CHROMATOGRAPHY ON ALUMINA (ACTIVITY IV) WITHOUT BINDER*

Estrogen	*EtAc*	*EtEther*	*AmAc*	*CCl_4-MeOH*			*CCl_4-EtOH*		*CCl_4-EtAc*	*CCl_4-PrOH*			*$CHCl_3$-MeOH*	
				(85:15)	(90:10)	(95:5)	(85:15)	(90:10)	(50:50)	(85:15)	(90:10)	(95:5)	(90:10)	(95:5)
Estrone	77	96	90	35	32	22	84	63	44	front	74	44	80	77
Estradiol-17β	57	85	84	26	22	16	74	48	28	89	63	31	80	63
Estriol	4	9	23	10	8	2	33	18	2	21	12	2	46	14
16-*epi*-Estriol	8	33	45	15	10	6	39	25	4	28	18	7	55	27
6α-Hydroxy-estradiol-17β	5	31	51	8	5	3	37	19	3	24	14	4	49	18
16-Oxo-estradiol-17β	24	48	68	22	20	12	64	41	10	66	44	19	77	54

Estrogen	*$CHCl_3$-EtOH*		*$CHCl_3$-EtAc*	*$CHCl_3$-PrOH*	*CH_2Cl_2-MeOH*	*CH_2Cl_2-EtOH*		*CH_2Cl_2-EtAc*	*CH_2Cl_2-PrOH*		*CH_2Cl_2-AmAc*	
	(90:10)	(95:5)	(90:10)	(95:5)	(90:10)	(95:5)	(90:10)	(95:5)	(90:10)	(90:10)	(95:5)	(75:25)	(50:50)
Estrone	88	80	35	78	front	70	95	76	38	95	81	70	73
Estradiol-17β	87	67	21	63	front	57	90	67	14	92	68	62	68
Estriol	33	11	1	6	67	7	32	14	1	20	7	6	10
16-*epi*-Estriol	49	30	3	18	75	18	47	32	1	40	17	19	23
6α-Hydroxy-estradiol-17β	40	15	1	9	68	9	42	20	1	31	11	7	14
16-Oxo-estradiol-17β	85	58	11	53	92	51	87	64	7	92	61	48	51

Estrogen	*$C_2H_3Cl_3$-MeOH*	*$C_2H_3Cl_3$-PrOH*	*$C_2H_4Cl_2$-MeOH*		*$C_2H_4Cl_2$-PrOH*	*C_6H_6-MeOH*		*C_6H_6-EtOH*		*C_6H_6-PrOH*	
	(95:5)	(95:5)	(90:10)	(95:5)	(95:5)	(90:10)	(95:5)	(90:10)	(95:5)	(90:10)	(95:5)
Estrone	29	58	68	65	64	48	25	51	32	76	57
Estradiol-17β	18	44	54	38	45	38	17	45	22	68	44
Estriol	3	3	28	6	4	18	2	22	2	14	4
16-*epi*-Estriol	7	12	36	13	11	25	6	25	6	21	8
6α-Hydroxy-estradiol-17β	5	4	25	8	7	19	4	23	4	18	5
16-Oxo-estradiol-17β	15	30	50	30	38	34	13	39	16	62	29

* CCl_4 = carbon tetrachloride; C_6H_6 = benzene; $C_2H_3Cl_3$ = trichloroethylene; $C_2H_4Cl_2$ = 1,2-dichloroethylene; CH_2Cl_2 = dichloromethane; $CHCl_3$ = chloroform; MeOH = methanol; EtOH = ethanol; PrOH = *n*-propanol; EtAc = ethyl acetate; AmAc = amyl acetate; EtEther = diethyl ether.

The R_F values in various solvent systems composed of halogenated hydrocarbons or benzene with the addition of alcohol, an ester or ether are listed in Tables I and II. Substitution of the estratrien, Δ^5-androstene and Δ^5-pregnene nucleus influences the R_F values in the usual manner as seen in adsorption chromatography on alumina. In estrogens, the mobility is decreased by the functional groups in the sequence: 16-ketone < 16β-hydroxyl < 6α-hydroxyl < 16α-hydroxyl and in the 3β-hydroxy-Δ^5-steroids: Δ^7-double bond < 17α-hydroxyl < 16α-hydroxyl < 21 hydroxyl < 7-ketone $\leqslant$ 7α-hydroxyl.

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Dünnschichtchromatographie von Aminozuckern auf Cellulosepulver

In den letzten Jahren wurden einige Verfahren zur Dünnschichtchromatographie von Zuckern bekannt. Während hierbei zunächst anorganische Schichten wie Kieselgel oder Kieselguhr[1] Verwendung fanden, wurde Cellulosepulver erstmals von SCHWEIGER[2] zur Trennung von Monosacchariden eingeführt. Nach diesen Ergebnissen schien es möglich, auch substituierte Zucker wie Glucosamin und Galactosamin und deren Acetylderivate auf Celluloseschichten zu trennen. In der vorliegenden Mitteilung wird über Versuche hierzu berichtet.

Die Platten wurden in bekannter Weise[2] mit dem Streichgerät der Fa. Desaga (Heidelberg) mit Cellulosepulver MN 300 der Fa. Macherey und Nagel (Düren, Deutschland) beschichtet (Schichtdicke 0.25 mm). Folgende Laufmittelgemische hatten sich bewährt:

I.	Butanol–Äthanol–Isopropanol–Ammoniak–Wasser	(2:4:0.5:0.5:1.5)
II.	Pyridin–Äthylacetat–Eisessig–Wasser	(5:5:1:3)
III.	Äthanol–Pentanol–Ammoniak–Wasser	(8:2:2:1)
IV.	Äthylacetat–Pyridin–Tetrahydrofuran–Wasser	(7:3:2:2)
V.	Äthylacetat–Isopropanol–Pyridin–Wasser	(7:3:2:2)

Gemische IV und V wurden bei Celluloseschichten angewandt, die mit Boratpuffer von pH = 8.0 (0.2 *M* Borsäure, 0.05 *M* NaCl und 0.05 *M* Borax = $Na_2B_4O_7 \cdot 10\ H_2O$) besprüht worden waren. Die Entwicklung der Chromatogramme dauerte etwa 2–3 Std. Eine Trennung von Glucosamin, Galactosamin, N-Acetylglucosamin und N-Acetylgalactosamin war in Systemen I, II und IV möglich (Tabelle I).

TABELLE I

VERGLEICH DER R_G-WERTE* IN VERSCHIEDENEN LAUFMITTELGEMISCHEN UND DER FARBNACHWEISE

Substanz	*R_G im Laufmittel*			*Färbung im TBS-Test***	*Färbung im U.V.-Licht*	*Nachweisgrenze (µg)*	
	I	*II*	*IV*			*TBS*	*Ninhydrin*
Glucosamin	1.00	1.00	1.00	gelb-orange	gelb	5	0.5
Galactosamin	0.91	0.83	0.88	hellbraun	grün	5	0.5
N-Acetylglucosamin	1.28	1.62	1.82	karminrot	rosa	3	—
N-Acetylgalactosamin	1.24	1.53	1.70	karminrot	rot	3	—

* $R_G = \frac{\text{Strecke in cm der Substanz}}{\text{Strecke in cm von Glucosamin}}$ nach zweimaligem Lauf.

** TBS-Test = Thiobarbitursäuretest (siehe Text).

Während sich Glucosamin und Galactosamin in den Systemen I und II voneinander trennen lassen, konnte bei der Trennung der acetylierten Verbindungen die Fähigkeit von Galactose zur Bildung von Boratkomplexen ausgenutzt werden.

Hierbei wurden die Platten vor dem Auftragen der Substanzen mit Boratpuffer besprüht, getrocknet und nach zweimaligem Lauf (je 3 Std.) erhielt man im System V die R_G-Werte in Tabelle II.

Die Zugabe von Flavognost (Diphenylborsäure-äthanolamin[3], Heyl, Hildesheim) zum Laufmittel hatte nur einen geringen Einfluss auf die R_G-Werte, die Flecke waren jedoch etwas kompakter. Die Trennung der N-Acetylderivate zeigt

TABELLE II

R_G-WERTE IM SYSTEM V (MIT BORATPUFFER)

Substanz	*R_G-Wert*
Glucosamin	1.00
N-Acetylglucosamin	2.95
N-Acetylgalactosamin	1.65

Fig. 1. Um eine Trennung aller vier Aminozucker gleichzeitig zu erreichen, wurde zweidimensional chromatographiert: zuerst im System II oder III, dann im System IV oder V. Nach zweimaligem Lauf im System II oder III färbt man einen Kontrollstreifen auf einer Seite der Platte an (siehe unten), besprüht danach bei Anwesenheit eines Fleckes in Höhe der N-Acetylverbindungen die Platte mit Boratpuffer. Nach weiterem zweimaligem Lauf im System IV oder V ergibt sich das in Fig. 2 und Fig. 3 dargestellte Bild. Die beste Trennung wird erzielt, wenn man die Platte vor dem ersten Lauf in jeder Richtung in der Kammer sättigen lässt.

Beim Nachweis der Aminozucker können entweder die Ninhydrinreaktion bei den nichtacetylierten Verbindungen oder der ELSON-MORGAN Test[4] bei den vier oben erwähnten Aminozuckern auf dem Cellulosepulver MN 300 A und der Thiobarbitursäuretest[5] auf dem Cellulosepulver MN 300 angewandt werden. Bei dem

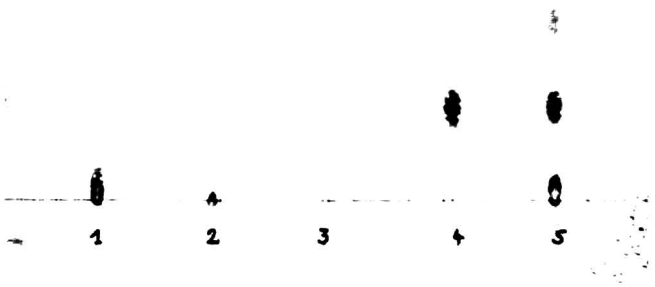

Fig. 1. 1 = Glucosamin; 2 = Galactosamin; 3 = N-Acetylglucosamin; 4 = N-Acetylgalactosamin; 5 = Mischung.

Cellulosepulver MN 300 A handelt es sich um ein von der Firma Macherey und Nagel (Düren, Deutschland) vorbehandeltes Cellulosepulver MN 300. Diese Vorbehandlung hat eine Herabsetzung der Aktivität zur Folge. Das Auftreten der charakteristischen Farbe beim ELSON-MORGAN Test wird auf dem Pulver MN 300 verhindert.

Die Anfärbung mit Hilfe des Thiobarbitursäuretestes[5] wurde folgendermassen durchgeführt. Die Schichten wurden nach dem Trocknen mit einer Lösung von 0.1 *M* Perjodsäure in Aceton (1.9 g HJO_4 in 10 ml H_2O, davon 1 ml + 19 ml Aceton), 10 Min. später mit einer Lösung von 3.5 %igem $NaAsO_2$ in 1 *N* HCl besprüht. Die braune Jodausscheidung verschwindet bei erneutem Besprühen. Nach 2 Min. wird die noch feuchte Platte mit einer 0.6 %igen alkoholischen Lösung von Thiobarbitursäure behandelt und 5 Min. bei 90° getrocknet.

Die beschriebene Methode weist einige Vorteile gegenüber der chromato-

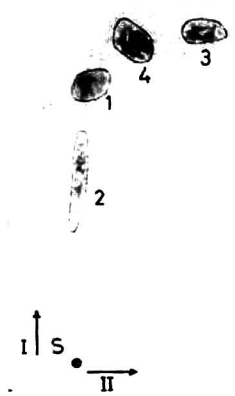

Fig. 2. 1 = Glucosamin; 2 = Galactosamin; 3 = N-Acetylglucosamin; 4 = N-Acetylgalactosamin. 1. Richtung: System III; 2. Richtung: System IV.

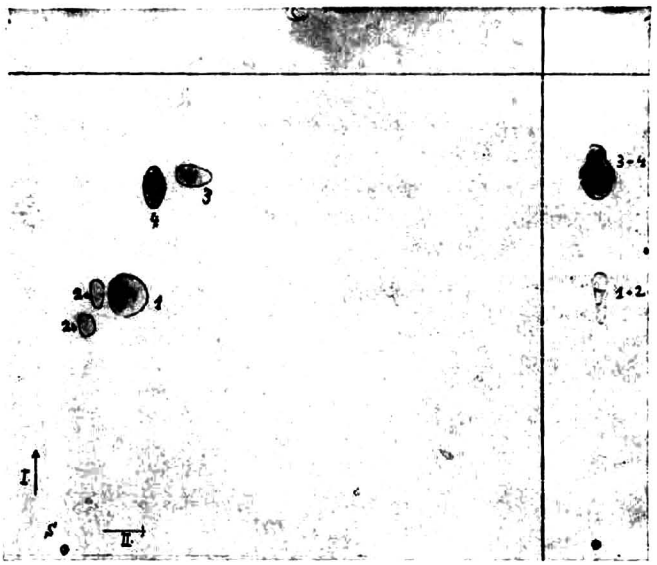

Fig. 3. 1 = Glucosamin; 2 = Galactosamin (2a = freie Base); 3 = N-Acetylglucosamin; 4 = N-Acetylgalactosamin. 1. Richtung: System II; 2. Richtung: System IV. Anfärbung im TBS-Test: Glucosamin = braun; Galactosamin = hellblau; N-Acetyl-Verbindungen = rot.

graphischen Trennung von Aminozuckern auf Papier auf. Während bei den bisherigen Verfahren Entwicklungszeiten von 24 Std. und mehr in Kauf genommen werden mussten[6,7], kann man auf der Cellulosedünnschicht eine Trennung in 4–6 Std. (bei zweidimensionaler Chromatographie in 6–8 Std.) erreichen. Auf Grund der kurzen Laufzeit treten Diffusionseffekte weniger stark in Erscheinung, es ergibt sich eine höhere Trennschärfe und endlich konnten durch die Auswahl der Laufmittel die bei Aminozuckern häufig auftretenden störenden Schwanzbildungen vermieden werden[8].

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Thin-layer chromatography of 2,4-dinitrophenylosazone homologs of vicinal dicarbonyls

Recent column partition[1,2] and adsorption[3] methods have facilitated the partial resolution of 2,4-dinitrophenylosazone (DNPO) mixtures. These methods have also been supplemented by a thin-layer chromatographic (TLC) technique that will separate the osazones of vicinal dicarbonyls into classes[4]. The purpose of this communication is to describe a TLC method that effects separation of a series of DNPO

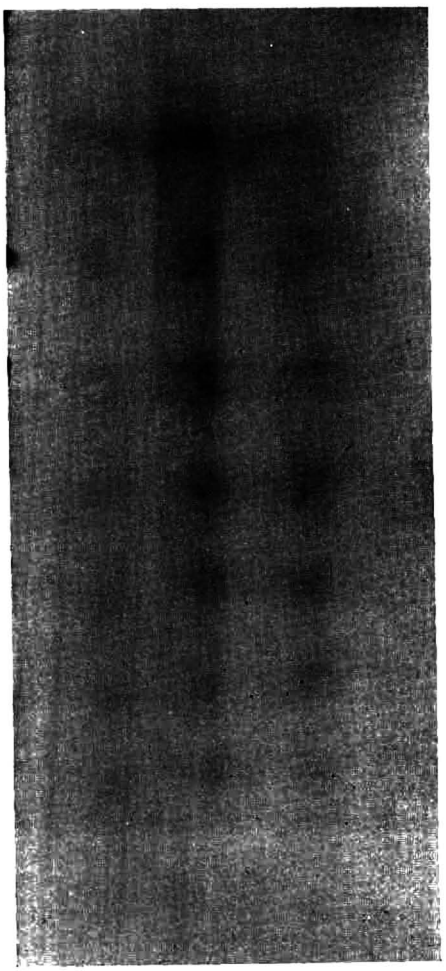

Fig. 1. Thin-layer chromatoplate of 2,4-DNPO of C_4–C_{10} α-ketoalkanals. Solvent front: 15 cm. Development time: 8 h.

homologs. The technique is a slight modification of a reversed-phase TLC system[5] for the separation of homologous series of 2,4-dinitrophenylhydrazones of monocarbonyl compounds.

Experimental

Thin-layer plates coated with silica gel G in a 250 μ layer were dried in an oven at 100° for 1 h, cooled, then dipped into a solution of 10% Shell Ondina 27* mineral oil in petroleum ether. Upon evaporation of the petroleum ether the plates were spotted with the DNPO mixture approximately $^1/_4$ in. from the base of the plate in the usual manner. The plates were developed for about 7 h (or until sufficient separation had been accomplished) in a system of dioxane–water (6:4). During development, one end of the plate was exposed to the atmosphere in the manner described by LIBBEY AND DAY[5]. This technique negates multiple development.

Fig. 1 illustrates the resolution of an homologous series of α-ketoalkanals. Since the system is reversed-phase chromatography, the higher the number of carbon atoms in the parent chain the slower the migration rate. 2,3-Diketones were also resolved by the technique. In our laboratory, a mixture of the C_4, C_5, C_7 and C_8 diketone derivatives, developed for 7 h, showed resolution approximating that of the α-ketoalkanals.

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* *Note added in proof.* Since submitting this note it has come to the authors' attention that Shell Oil Company no longer manufactures mineral oils. Nujol, a product of Plough, Inc., New York, has been evaluated as a substitute stationary phase and found to perform adequately. It is assumed that comparable grades of mineral oil also may be employed.

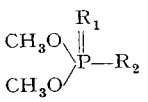
Zum Nachweis systemischer Thiophosphorsäureester vom Typ „Systox" und deren oxydierten Analogen

Die Rückstandsanalyse der Thiophosphorsäureester vom Typ "Systox" erfordert wegen des Um- und Abbaues dieser Insektizide und der dabei auftretenden Zwischenprodukte besonders empfindliche Nachweisreaktionen.

Das Insektizid Tinox enthält als Wirkstoff O,O-Dimethyl-O-äthylmercaptomethyl- (I) und O,O-Dimethyl-S-äthylmercaptomethyl-thiophosphat (II). Durch Einwirkung von Licht, Luftsauerstoff und pflanzeneigenen Enzymen ist einmal eine Isomerisierung der Verbindungen I zur Thiolverbindung II möglich, zum anderen können die Verbindungen I und II zu Sulfoxid und Sulfon oxydiert werden.

TABELLE I

THIOPHOSPHORSÄUREESTER VOM TYP "SYSTOX" UND ANALOGEN



Verbindung	R_1	R_2
I (Thiono-Tinox)	=S	$-OCH_2CH_2SCH_3$
II (Thiol-Tinox)	=O	$-SCH_2CH_2SCH_3$
III (I-Sulfoxid)	=O	$-OCH_2CH_2SO\cdot CH_3$
IV (I-Sulfon)	=O	$-OCH_2CH_2SO_2\cdot CH_3$
V (II-Sulfoxid)	=O	$-SCH_2CH_2SO\cdot CH_3$
VI (II-Sulfon)	=O	$-SCH_2CH_2SO_2\cdot CH_3$

Untersuchungen mit diesen Verbindungen gaben eindeutig zu erkennen, dass mit den üblichen Nachweisreagenzien wie Kaliumhexajodoplatinat[1], Palladiumchlorid[2], Jodazid[3], 2,6-Dichlorchinonchlorimid[4], Silbernitrat–Bromphenolblau[5] bzw. Brom–Fluorescein[6] nicht alle darstellbaren Oxydationsprodukte nachweisbar sind bzw. dass sie nicht in den zu erwartenden Mengen sichtbar reagieren. Dies trifft vor allem für die Sulfone IV und VI sowie die mit Wasserstoffperoxid oxydierte Verbindung III des Thiono-Tinox (O,O-Dimethyl-O-äthylsulfoxylmethylphosphat) zu.

Um auch diese bisher chemisch auf direktem Wege nicht oder nur schwer erfassbaren Verbindungen auf dem Dünnschichtchromatogramm in Mengen unter 10 μg mit einfachen Reaktionen nachweisen zu können, wurden von uns Kaliumpermanganat und Kobalt(II)chlorid[7] als Sprühreagenzien geprüft.

Dünnschichtchromatographische Auftrennung

Für die vollständige Auftrennung der verschiedenen Metabolite verwendeten wir die Dünnschichtchromatographie mit Kieselgel G "Merck" oder Kieselgel B "Wolfen" als Adsorbens und als Laufmittel ein von uns bereits früher beschriebenes System (WS 3), bestehend aus Toluol–Methanol–Isopropanol–Acetonitril–Wasser (40:16:16:20:9)[8]. Mit diesem System wird ein guter Trenneffekt erzielt, wobei alle Verbindungen in gut begrenzten Flecken wandern und für die Auswertung ent-

sprechend weit voneinander getrennt werden. Beide Adsorbentien sind für die Chromatographie geeignet, der Trenneffekt ist jedoch beim Kieselgel B durch die grösseren Differenzen zwischen den R_F-Werten der verschiedenen Metabolite besser (Tabelle II).

TABELLE II

R_F-WERTE DER UNTERSUCHTEN THIOPHOSPHORSÄUREESTER IM SYSTEM WS 3

Thiophosphorsäureester	*R_F-Wert*	
	*Kieselgel B**	*Kieselgel G***
Thiol-Tinox (II)	0.69	0.81
Thiono-Tinox (I)	0.81	0.86
Thiol-Tinox-Sulfoxid (V)	0.42	0.54
Thiono-Tinox-Sulfoxid (III)	0.48	0.68
Tinox-Sulfon (IV, VI)	0.63	0.80
Thiol-Systox	0.71	0.83
Thiono-Systox	0.84	0.89
Thiol-Systox-Sulfoxid	0.58	0.71

* Kieselgel B: VEB Farbenfabrik, Wolfen.
** Kieselgel G: E. Merck A.G., Darmstadt.

Die Nachweisempfindlichkeit wird von den verwendeten Kieselgelen nicht unterschiedlich beeinflusst. Die Laufzeit beträgt bei Zimmertemperatur und 10 cm Steighöhe für Kieselgel G 20–25 Min., für Kieselgel B 45–50 Min. Die Nachweisgrenze liegt für die Verbindungen I, II und V bei 0.5–1 μg/Fleck.

Das oben erwähnte Laufmittelsystem ist auch für die Papierchromatographie geeignet. Durch den Wasserzusatz erübrigt sich ein vorausgehendes Äquilibrieren des Papiers. Bei einer Laufstrecke von 20 cm (absteigend) beträgt die Laufzeit 4–5 Std. bei Verwendung von "Schleicher & Schüll 2043b". Die Empfindlichkeit ist bei der Papierchromatographie jedoch etwas geringer (Nachweisgrenze für I, II und V 2–3 μg). Ausser der Empfindlichkeit wird auf dem Papier auch die Trennung der Umwandlungsprodukte etwas beeinträchtigt.

Nachweis

Das Sichtbarmachen der Flecken mit den bereits in der Literatur für andere Thiophosphorsäureester beschriebenen Sprühreagenzien erfolgte nach den Originalvorschriften. In unseren Untersuchungen erwies sich besonders das *Kaliumhexajodoplatinat* zum Nachweis der Thiono- und Thiol-Isomeren sowie des Thiolsulfoxids als gut geeignet. Die Färbung tritt bei den Verbindungen I und II sofort ein, während die optimale Ausfärbung von V nach ca. 30 Min. erreicht wird. Die Verbindung VI lässt sich mit etwas geringer Empfindlichkeit nach einigen Stunden ebenfalls nachweisen.

Die gelbe Färbung mit Kaliumhexajodoplatinat auf rosa Hintergrund gestattet eine gute Erkennung und Auswertung der Flecken. Eine beschränkte Stabilisierung (für ca. 1–2 Tage) der normalerweise nach wenigen Stunden verblassenden Färbung wird durch kräftiges Besprühen und sofortige Einwirkungen von hellem Licht erzielt; der Hintergrund verfärbt sich dabei nach graurosa.

TABELLE III

FARBE DER FLECKE NACH BESPRÜHEN

Es wurden jeweils 30 μg angewendet.

Thiophosphorsäureester	K_2PtJ_6	$PdCl_2$	*Jodazid (+ Stärkelsg.)*	$CoCl_2$ *in Aceton*	$KMnO_4$ *in Aceton*	*2,6-Dichlorchinonchlorimid*	$AgNO_3$–*Bromphenolblau*
Thiol-Tinox (II)	gelb	gelb	weiss	olivgrau	gelb	gelb	—
Thiono-Tinox (I)	hellgelb	gelb	weiss	olivgrau	gelb	braunorange	braun
Thiol-Tinox-Sulfoxid (V)	hellgelb-weiss	gelb	—	olivgrau*	gelb	hellgelb	—
Thiono-Tinox-Sulfoxid (III)	dunkelgelb**	—	fraglich	olivgrau	gelb	violettgrau	—
Tinox-Sulfon (IV, VI)	hellgelb	—	—	rotbraun***	gelb	braunorange	braun
Thiol-Systox	gelb	gelb	weiss	nicht unters.	gelb	orange	—
Thiono-Systox	hellgelb	gelb	weiss	nicht unters.	gelb	braunoranges	braun
Thiol-Systox-Sulfoxid	hellgelb-weiss	gelb	—	nicht unters.	gelb	nicht unters.	—
Hintergrund	rosa	weiss	blau	blau	rosa	weiss	blau

* Erscheint nach Erhitzen 30–60 Min. auf 150°.
** Nur schwach erkennbar.
*** Erscheint langsam nach Erhitzen 1 Std. auf 150°.

Für den Nachweis der Verbindungen I und II lässt sich mit annähernd gleicher und für V mit etwas verringerter Empfindlichkeit auch das Palladiumchlorid in schwach salzsaurer Lösung verwenden. Die Verbindung VI spricht — zumindest in Mengen unter 30 μg Fleck — auf Palladiumchlorid nicht an.

Mit 2,6-Dichlorchinonchlorimid, Jodazid, Silbernitrat-Bromphenolblau und Kobaltchlorid erreicht man — soweit eine Farbbildung überhaupt auftritt — nicht die Empfindlichkeit der beiden erstgenannten Nachweisreagenzien.

Wie aus Tabelle III zu ersehen ist, bereiten besonders die Oxydationsprodukte der Thiono-Isomeren (III und IV) beim Nachweis mit den oben genannten üblichen Sprühreagenzien Schwierigkeiten. Eine eindeutige Anzeige dieser Verbindungen mit diesen Sprühreagenzien ist in Mengen unter 30 μg nicht möglich.

ERTEL UND HORNER[9] verwenden zum Nachweis von Phosphinoxiden Kaliumpermanganat in konz. Schwefelsäure. Das gleiche Reagenz benutzten auch MASTRJUKOWA, SACHAROWA UND KOBATSCHNIK[10] zum Nachweis von Thiono- und Thiol-Systox sowie einigen anderen Thiophosphorsäureestern. Für den Nachweis der Verbindungen I bis VI in Mengen unter 30 μg ist dieses Reagenz jedoch nicht geeignet.

Eine sofortige Reaktion kleinster Mengen des Tinox und seiner Umwandlungsprodukte bei einer gleichzeitig hohen Empfindlichkeit konnten wir mit einer 0.2 %-igen acetonischen Kaliumpermanganatlösung erzielen. Selbst die mit keinem anderen Sprühreagenz in genügender Empfindlichkeit nachweisbaren Verbindungen III und IV lassen sich mit diesem Sprühreagenz noch in der Grössenordnung von 1–2 μg eindeutig erkennen.

Als Nachteil dieses Verfahrens muss die sehr geringe Haltbarkeit der mit Permanganat erhaltenen Färbung angesehen werden. Eine Stabilisierung für 30–60 Min. lässt sich erreichen, wenn das Chromatogramm während des Verblassens des Hintergrundes ein zweites Mal besprüht wird.

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* Leiter: Dr. R. ENGST.

Some observations on quantitation in temperature programmed gas chromatography

The possibilities of quantitative analysis by gas chromatography are well known and have been described often in the literature and in text books. Reports on the application of quantitative gas chromatography to a specific problem are not, however, so frequent and very little can be found in the literature on the quantitative aspects of programmed gas chromatography.

In this paper the use is discussed of gas chromatography to investigate the Grignard addition reaction. In this investigation the relative reactivity of phenylmagnesium bromide towards acetophenone and derivatives was measured[1] using temperature programmed gas chromatography.

Equimolecular amounts of acetophenone and of a series of substituted acetophenones were allowed to react with an insufficient amount of phenylmagnesium bromide in diethyl ether and with diphenyl ether as internal standard.

By estimation of the surface of the gas chromatographic peaks of the recovered ketones, in relation to the surface of the diphenyl ether peak, the ratio of the reactivities is obtained. The same ratio can be determined by gas chromatographic determination of the amounts of reaction products. Comparison of the results obtained in the two ways showed excellent agreement and a Hammett plot was obtained with $\rho = 0.415$. The composition of the reaction mixture was rather complex and had a large boiling range.

Each mixture contained acetophenone (b.p. 202°), the acetophenone derivative (b.p. 200–310°), the internal standard diphenyl ether (b.p. 259°), the addition product of the acetophenone derivative with phenylmagnesium bromide (b.p. 250–400°), the addition product of acetophenone with phenylmagnesium bromide, diphenyl-methyl-carbinol (b.p. 260°), by-products of the Grignard reaction, *e.g.* phenol (b.p. 182°), methyl-phenyl-carbinol (b.p. 202–204°), biphenyl (b.p. 255°), and of course solvents such as ether and benzene. The by-products were identified after their separation from the reaction mixture by preparative gas chromatography (Aerograph 700).

The wide boiling range of the mixtures demands temperature programmed gas chromatography.

Separations in this way are perfectly possible and an advantage of the programming is that all peaks have nearly the same shape with sharp slopes showing little or no tailing. This is important for the ball and disc integrator which was used for integration. In isothermal chromatography the conditions can easily be kept constant during a run and differences between several analyses are eliminated by using an internal standard. With programmation the gas rate changes easily because of the increase of gas viscosity with temperature and although this could be standardised it should preferably be independent of starting temperatures and programming rates. This condition was fulfilled using an apparatus consisting of a dual column Aerograph 350-B (Wilkens Instruments) with thermal conductivity detectors in a separate oven, Moore differential flow controllers and needle valves. These allow a large pressure differential to be applied at the column inlet and are indeed necessary to obtain a constant gas flow through the detector cell regardless of programming rate or temperature. With three component mixtures it was found under these conditions that

the ratio of the peak areas was the same when programming from 40° onwards at 4°/min or when starting at 60° with a 6°/min heating programme.

Even without pressure drop at the column inlets and thus with a variable gas rate, reproducibility can be very good. This is shown in Table I, obtained for aceto-

TABLE I

No. of analysis	*Substance*	*Temperature of emergence (C)*	*Relative surface*	*Gas flow rate*
1	Acetophenone	190	0.6757	11.5
	Diphenyl ether	233	1	13.0
	Acetonaphthone	253	1.0434	13.8
2	Acetophenone	192	0.6863	11.5
	Diphenyl ether	234	1	13.0
	Acetonaphthone	253	1.0354	13.8–14.0
3	Acetophenone	191	0.6787	11.4
	Diphenyl ether	234	1	13.0
	Acetonaphthone	253	1.0505	13.8–14.0

phenone, diphenyl ether and acetonaphthone with a programme rate of 8°/min and a starting temperature of 170°. The gas flow is given as time in seconds for 10 ml. In general, however, instrument stability is much better with a pressure drop at the column inlets.

The high temperature necessary for the analyses restricted the choice of stationary phase. The use of combined columns with SE30 and Apiezon L made possible the analysis of all the mixtures. SE30 separates diphenyl ether and biphenyl but separation of acetophenone and the carbinols is not so good. Apiezon L gives excellent separation of the acetophenones and the carbinols, but diphenyl ether and biphenyl are not separated. Analysis on SE30 then gives the correction necessary in the analyses on Apiezon L. Quantitation of the results was obtained with a disc integrator. The accuracy of this device is excellent and was in our case $\pm$ 0.15 %. The analysis of each mixture was repeated until at least 9 values for the relative reactivity were obtained. The maximum deviation between the values was *ca.* 5 % and the deviation of the mean value from the Hammett plot was at most 1 %. To obtain this accuracy, the baseline on the chromatogram has to remain constant to 0.1 % of the total deflection, otherwise the integrated peak surfaces are valueless. This can be obtained on a not too sensitive attenuation setting (4 $\times$ or 8 $\times$) when the stationary phase is stable and when no decomposition occurs.

Frequently, however, after the emergence of a peak, the baseline did not come back to absolute zero, but showed a shift of 0–2 mm. The reason for this is unknown. Although hardly noticeable at first glance this had to be corrected for the quantitation by manual setting of the instrument so that the chromatograms needed constant attention. An interesting point about possible decomposition is that diphenylmethyl-carbinol was destroyed on SE30 but not on Apiezon L although all other conditions were maintained identical. After prolonged use the Apiezon L columns also destroyed this alcohol.

Because of the high cost of helium in Europe the possible use of hydrogen as carrier gas for quantitative work was investigated. Curiously enough the accuracy of the results was not as good as with helium, the deviation on relative areas being about three times as great. It was at first thought that a possible reaction on the heated wires (hydrogenation) was responsible. However, the relative surfaces of a mixture of toluene and *trans/cis*-decalin was the same using helium or hydrogen. This makes hydrogenation or dehydrogenation on the detector wires improbable. The reason for the better results with helium than with hydrogen is unknown to us. The stability of the instrument seems to be better with helium than with hydrogen. For very precise quantitative work with katharometer detection, therefore, it seems that hydrogen is less suitable than helium.

An example of a chromatogram is shown in Fig. 1a, obtained with a 2 m column filled with 15 % SE30 on celite. The programming was 6°/min and the starting tem-

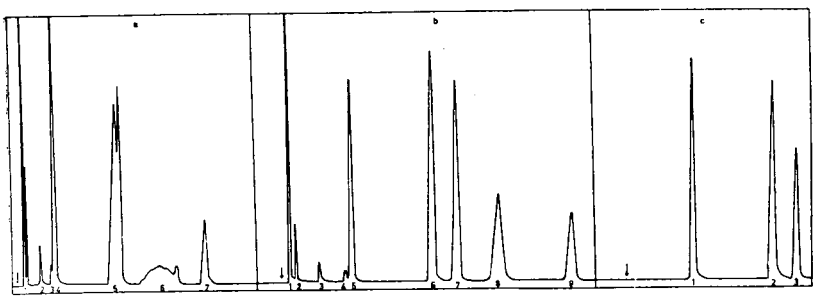

Fig. 1. Separation by temperature programmed gas chromatography of a competitive Grignard reaction mixture as explained in the text (Aerograph 350-B).

perature 136°. The order of emergence is ether and benzene (1), phenol (2), methyl-phenyl-carbinol (3), acetophenone (4), 4-methoxyacetophenone, biphenyl and diphenyl ether in a composite peak (5), methyl-diphenyl-carbinol with decomposition (6) and 4-methoxyphenyl-methylphenyl carbinol (7). The chromatogram of Fig. 1b shows the same separation on a 2 m column with 15 % Apiezon L on celite. The same operational conditions ware maintained. The order of emergence is ether (1), benzene (2), phenol (3), methyl-phenyl-carbinol (4), acetophenone (5), 4-methoxyacetophenone (6), biphenyl and diphenyl ether (7), methyl-diphenyl-carbinol (8) and 4-methoxyphenyl-methylphenyl-carbinol (9). The chromatogram of Fig. 1c is obtained on the same column and under the same conditions as Fig. 1b and shows the separation of an equimolecular mixture of acetophenone (1), 4-methoxyacetophenone (2) and the internal standard diphenyl ether (3) to which the Grignard reagent was added. From the surfaces relative to the diphenyl ether peak in Fig. 1b and 1c the relative reactivity of acetophenone and 4-methoxyacetophenone can be calculated.

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Ein papierchromatographisches Trennverfahren für Lipoproteide

Die zeitraubenden Versuche bei der Untersuchung von Lipoproteiden, sowie die schwachen Trenneffekte die man mit der Papierelektrophorese erzielen kann, machten es nötig für diese Stoffe ein geeignetes chromatographisches Trennverfahren auszuarbeiten. Die bisher in Gebrauch befindlichen papierchromatographischen Trennverfahren für Lipoproteide wenden unbequeme, oft schädlich wirkende, Laufmittel an, mit denen kaum gute Trenneffekte zu erzielen sind[1,2].

Das Ziel unserer Forschungsarbeiten war, ein neues Kriterium für die Beurteilung von Lipoproteiden im Blutserum in der klinischen Praxis auszuarbeiten. In einer Reihe von Versuchen gelang es uns ein einfaches und unserem Erachtens nach, sehr effektives papierchromatographisches Trennverfahren auszuarbeiten. Ein Kurzbericht über die erzielten Resultate wurde auf dem Kongress der Gesellschaft Polnischer Gynäkologen in Gdańsk[3-5] und auf dem I. Biochemischen Kongress in Łódź[6] vorgetragen. Nach unserem Verfahren werden die Lipoproteide mit Hilfe der eindimensionalen Papierchromatographie aufsteigend getrennt, wobei als Trägerstoff das Papier Whatman Nr. 1 und als mobile Phase destilliertes Wasser verwendet werden.

Bei der experimentellen Ausführung unseres Verfahrens wird folgendermassen vorgegangen. Entsprechend gekennzeichnete Papierstreifen von 25 cm Länge werden in einer chromatographischen Kammer mit dem unteren Rand 2 cm tief in destilliertes Wasser eingetaucht. Nachdem die Lösungsmittelfront den oberen Rand des Papierstreifens erreicht hat, wird dieser noch für 10 Min. in der Kammer gelassen. Bei diesem Vorgang werden die Papierstreifen gleichmässig angefeuchtet und zum Teil ausgewaschen.

Die Papierstreifen werden nachher in einer gleichen Kammer, jedoch ohne Wasser, bei Raumtemperatur zum trocknen aufgestellt. Nach etwa 45 Min. werden mit einer Mikropipette oder kalibrierten Kapilare, 4 cm vom unteren Rand entfernt, auf eine 5 cm lange Startlinie 0.02 ml des zu untersuchenden Blutserums aufgetragen. Die startfertigen Papierstreifen werden in eine mit destilliertem Wasser gefüllte Entwicklungskammer gebracht, wobei der untere Rand der Papierstreifen 2 cm tief ins Wasser eingetaucht wird. Nachfolgend wird bei geschlossener Kammer entwickelt. Nach einer Stunde werden die Papierstreifen herausgenommen und bei Raumtemperatur getrocknet.

Auf dem trockenen Chromatogramm werden die Lipide mit Sudanschwarz nach SWAHN[7] sichtbar gemacht. Zur quantitativen Bestimmung der Lipide werden die blaugefärbten Zonen aus dem Papierstreifen herausgeschnitten und mit 4 ml einer Äthylalkohol-Eisessigsäure-Mischung (4:1) eluiert. Die Extinktion des Eluats wird spektrophotometrisch bei $\lambda = 590$ mμ bestimmt. Als Eichlösung dient dieselbe Mischung mit der ein ungefärbter Teil der gleichen Grösse, wie der der gefärbten Zone, aus demselben Streifen eluiert wurde. Auf diesem Wege kann das Verhältnis der einzelnen Lipoproteidfraktionen zueinander, sowie der gesamte Lipidgehalt in der untersuchten Probe bestimmt werden.

Besprechung der Versuche

Bei Anwendung unseres Verfahrens zur Trennung der Lipoproteide aus menschlichem Blute werden zwei (Fig. 1) bis drei (Fig. 2 a, c und d) gut getrennte Fraktionen erhalten. Im Blutserum einiger kranker Personen konnte ausserdem noch eine vierte,

zwischen dem ersten und zweiten Streifen liegende Fraktion festgestellt werden. Diese Fraktion konnte jedoch nicht in jedem Versuch festgestellt werden, manchmal sogar auch nicht bei wiederholter Trennung desselben Serums.

Bei lipemischen Blutseren und Seren mit Glycerintrioleatzusatz blieb auf der Startlinie eine mehr oder wenig scharf angedeutete, aus freien Lipiden bestehende Zone zurück (Fig. 2b). Aus technischen Gründen gelang es uns einstweilen noch nicht

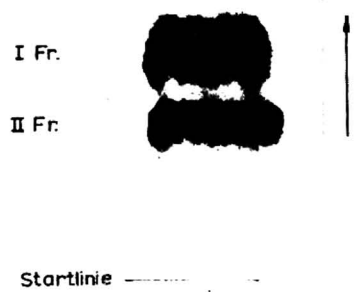

Fig. 1. Chromatogramm des normalen Serums.

die einzelnen Fraktionen zu identifizieren. Wir stellten jedoch fest, dass β-Lipoproteid-freie Seren sich nicht in der Streifenzahl vom normalen Serum unterscheiden. Dieser Befund wurde mit einem β-Lipoproteidfreien Serum erhalten, aus dem das β-Lipoproteid nach WIEME[8] entfernt wurde, sowie mit einem Serum aus Nabelblut, dass wie bekannt, beinahe β-Lipoproteidfrei ist[9] (Fig. 2c und d). Ein nach BURSTEIN UND SAMAILLE[10] erhaltenes β-Lipoproteid zeigte auf dem Chromatogramm nur eine Zone (Fig. 3a) und zwar mit dem gleichen R_F-Wert wie der der zweiten Serumfraktion (Fig. 3b). Hinter dieser Fraktion zieht sich ein farbiger Schwanz der bis zur Startlinie reicht, auf der auch noch deutliche Lipidspuren sichtbar sind. Diese Erscheinung kann als Zerfall des Lipoproteids gedeutet werden. Auf dem auf Fig. 3c sichtbaren

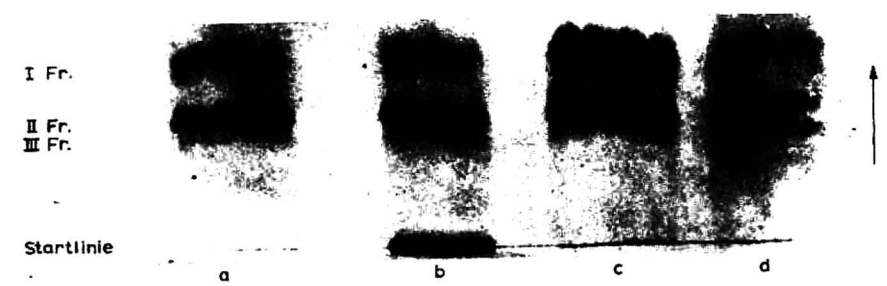

Fig. 2. Chromatogramm der Sera. (a) Normales Serum; (b) Normales Serum mit Trioleatzusatz; (c) Mutterserum; (d) Nabelschnurblutserum.

Chromatogramm wurde eine mit der Ultrazentrifuge erhaltene, α-Lipoproteide enthaltene Fraktion, getrennt. Auf diesem Chromatogramm sind drei farbige Streifen erkennbar, die etwa den Lipoproteidfraktionen des Blutserums entsprechen. Die angeführten Beispiele zeigen, dass nach unserem Verfahren sehr einfach und mit gutem Erfolg Lipoproteidmischungen getrennt werden können. Dieses Verfahren kann auch zur Trennung von Lipoproteiden aus anderem biologischen Material verwendet werden.

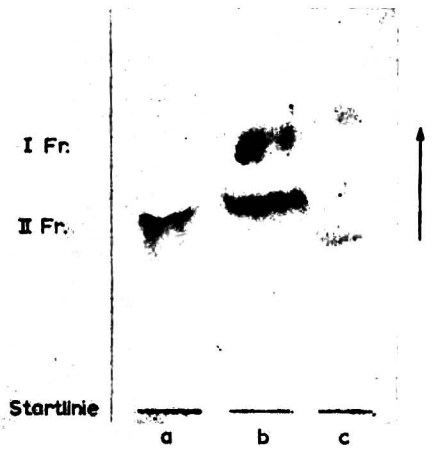

Fig. 3. Chromatogramm von Lipoproteiden. (a) β-Lipoproteid nach BURSTEIN UND SAMAILLE; (b) Serumlipoproteide; (c) α-Lipoproteide aus der Ultrazentrifuge.

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Herrn Ing. M. SOBESLAVSKY aus dem Ustav Hematologie a Krevni Transfuze in Prag danken wir bestens für die Überlassung von Lipoproteidpräparaten.

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Direkte kolorimetrische Bestimmung von Alkaloiden aus Papyrogrammen

In der Literatur sind zahlreiche Methoden zur Bestimmung von Alkaloiden nach papierchromatographischer Trennung beschrieben. Die densitometrische oder planimetrische Auswertung der Chromatogramme führt nicht in allen Fällen zu befriedigenden Ergebnissen; die Voraussetzung ist eine regelmässige geometrische Form der Flecke. Zudem muss im allgemeinen für jede Bestimmung eine Standardreihe mit ausgewertet werden.

Häufiger werden daher Verfahren angewendet, die auf einer Elution der Chromatogramme beruhen. Die Lokalisierung der Flecke vor der Bestimmung bereitet bei nicht fluoreszierenden Verbindungen oft Schwierigkeiten und ist auch mit Hilfe von Leitchromatogrammen nicht immer einwandfrei durchzuführen.

Die günstigen Voraussetzungen bieten also Methoden, die eine Bestimmung der Substanzen nach Behandlung mit einem Anfärbereagenz gestatten. Nach Anwendung von Dragendorff-Reagenz können die Flecke eluiert und kolorimetrisch bestimmt werden[1]. Die gleichmässige Durchfeuchtung des Papieres mit dem Reagenz bereitet aber mitunter Schwierigkeiten. ROMEIKE[2] umging bei der Bestimmung der Tropa-Alkaloide diesen Nachteil. Sie zerlegte den Dragendorff-Komplex mit Ammoniak und reinigte die Basen durch mehrfaches Umschütteln vor Durchführung der kolorimetrischen Reaktion. Abgesehen von dem grösseren Arbeitsaufwand versagt diese Methode bei quartären Salzen.

Wir verwenden zur Bestimmung der Alkaloide nach Ausbleichen der Dragendorff-Flecke mit Ammoniak die Indikator-Extraktionsmethode[3]; sie beruht auf der Umsetzung der Basen mit Indikatoren bei einem definierten pH-Wert und Extraktion des Komplexes mit einem organischen Lösungsmittel. Bei Verwendung von Bromthymolblau und Citrat-Puffer, pH 7.5, kann eine grosse Anzahl von Alkaloiden kolorimetrisch bestimmt werden. Die Auswertung erfolgt mit Hilfe einer Eichkurve, die direkt an Reinsubstanz erstellt wird.

Wir konnten aus Papierchromatogrammen und Elektropherogrammen Lobelin, Atropin, Codein und Buscopan (N-Butyl-hyoscinbromid) auch in Gemischen bei routinemässiger Ausführung mit einer Reproduzierbarkeit von $\pm$ 2 % bestimmen.

Ausführung

Die Probelösung wird mit einer Hamilton-Spritze wie üblich auf der Startline aufgetragen. Als Fliessmittel muss ein Amin-freies Gemisch (NH_3 stört nicht) verwendet werden. Das Trocknen der Chromatogramme erfolgt unter Schutz vor Amin-Dämpfen. Nach dem Trocknen wird mit modifiziertem Dragendorff nach MUNIER UND MACHEBOEUF besprüht und die Substanzflecke mit Bleistift markiert. Der Bogen wird dann in eine Kammer gebracht, die mit Ammoniak-Dämpfen gesättigt ist. Die Flecke bleichen in wenigen Minuten aus, werden dann ausgeschnitten, zerkleinert und in einem Scheidetrichter mit 20 ml Pufferlösung, pH 7.5, einige Minuten geschüttelt. Nach Zugabe von 1 ml Bromthymolblaulösung wird 3 Mal je 20 ml Chloroformlösung ausgeschüttelt. Die organischen Phasen werden in einem 100 ml Messkolben gesammelt, in dem 25 ml Borsäurelösung vorgelegt sind. Zur Abtrennung von Papierfasern und geringen Anteilen der wässrigen Phase können die Chloroformphasen durch ein kleines Silikonfilter (Schleicher & Schüll 595 $^1/_2$ HY) filtriert werden. Der Messkolben wird dann mit Äthanol zur Marke

aufgefüllt und die Extinktion der Farblösung in 2 cm Küvetten bei 436 nm gegen die Blindausschüttelung eines Papierstückchens von etwa gleicher Grösse wie der Substanzfleck aus dem gleichen Papyrogramm gemessen. Die Farblösung ist stabil. Zur Auswertung wird eine Eichkurve durch Einwaage von Reinsubstanz und Messung gegen einen Reagenzienblindwert erstellt (Fig. 1).

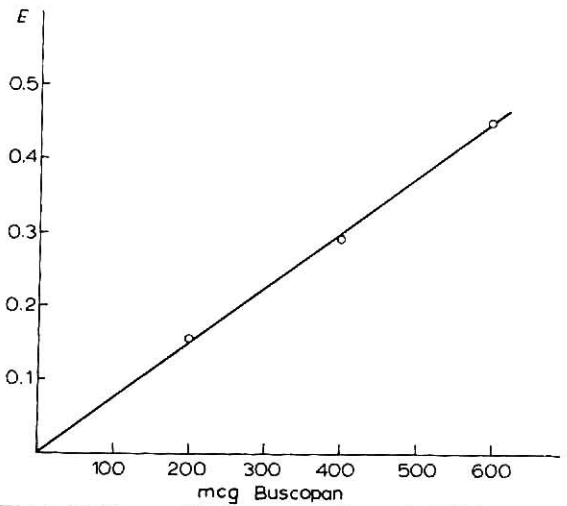

Fig. 1. Eichkurve für Buscopan. Eppendorf-Fotometer, Filter Hg 436, 2 cm Küvetten. O = Werte nach papierchromatographischer Trennung.

Reagenzien

Pufferlösung pH 7.5: 7.5 ml 0.1 *M* Citronensäurelösung werden mit 0.2 *M* Dinatriumphosphatlösung auf 100 ml aufgefüllt.

Bromthylmolblaulösung: 0.15 g Bromthymolblau und 0.15 g wasserfreies Natriumcarbonat werden in Wasser gelöst und auf 100 ml aufgefüllt.

Borsäurelösung: 5.0 g Borsäure werden mit Alkohol und 20 ml Wasser unter Erwärmen zur Lösung gebracht und mit Äthanol auf 250 ml aufgefüllt.

Äthanol ca. 96 %ig: die mit den üblichen Vergällungsmitteln (Benzol, Toluol etc.) behandelte Qualität ist verwendbar.

Chloroform: DAB-Qualität.

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Ein Verfahren der zweidimensionalen quantitativen Papierchromatographie mit Eichstandards auf dem selben Papierbogen

Zur quantitativen papierchromatographischen Bestimmung von Aminosäuren wurde eine grosse Zahl von eindimensionalen[1-7] und zweidimensionalen[8-12] Verfahren entwickelt. Während die eindimensionale Papierchromatographie den Vorteil hat, dass leicht Vergleichsstandards auf dem selben Papierbogen aufgetragen werden können, ist es sehr nachteilig, dass etwa sechs Laufmittelsysteme gebraucht werden, um die in biologischem Material vorkommenden Aminosäuren auftrennen zu können. Beim zweidimensionalen Verfahren mit seinem weitaus grösseren Auflösungsvermögen muss man sich mit generell aufgestellten Eichkurven begnügen. Selbst unter möglichst konstanten Versuchsbedingungen ist es aber recht schwierig, eine gut reproduzierbare Farbentwicklung zu erreichen. Zu viele unkontrollierbare Einflüsse, wie Wassergehalt des Papiers[4], Laufmittelrückstände im Papier[4], Wasserdampfdruck im Heizofen[4], und Oxydationszustand des Ninhydrinreagens[7] beeinträchtigen die Reproduzierbarkeit.

Um die Vorteile beider Methoden, nämlich hohes Auflösungsvermögen und individuelle Eichkurven für jedes Chromatogramm, zu kombinieren, werden auf etwa 65 × 65 cm grossen Bogen vier zweidimensionale Chromatogramme gleichzeitig hergestellt. Während ein oder zwei Starpunktet das zu untersuchende Gemisch auf-

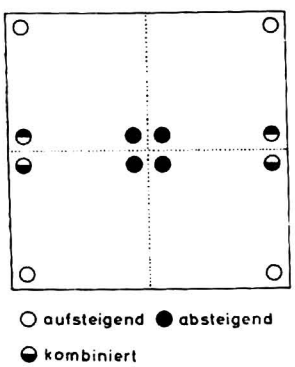

Fig. 1.

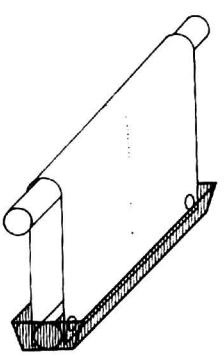

Fig. 2a.

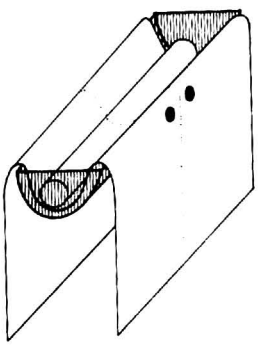

Fig. 2b.

Fig. 1. Lage der Auftragstellen für aufsteigende und absteigende Chromatographie, sowie für die Kombination beider Verfahren.

Fig. 2. Anordnung der Papiere im Trog bei aufsteigender und absteigender Chromatographie.

nehmen, dienen die zwei bzw. drei verbleibenden Startpunkte zum Auftragen des Vergleichsgemisches. (Zur Aufstellung einer Eichkurve bei spektralphotometrischer Auswertung des Ninhydrinkupferkomplexes sind minimal zwei Werte nötig[4].) Je nach Lage der Startpunkte ist aufsteigende und absteigende Chromatographie, sowie eine Kombination beider Verfahren möglich (Fig. 1). Für aufsteigende Chromatographie wird das Blatt an einer Mittellinie gefaltet und über einen Glasstab gehängt (Fig. 2a). Die beiden herunterhängenden Seiten tauchen in die Wanne mit dem Laufmittel ein. Ein Glasstab in der Wanne verhindert, dass die beiden Seiten zusammenkleben. Für

absteigende Chromatographie wird der Bogen gemäss Fig. 2b in eine Glaswanne eingelegt und mit einem Glasstab festgehalten. Nach dem ersten Lauf und Abdampfen des Laufmittels wird der Bogen senkrecht zur ersten Richtung gefaltet und der zweite Lauf erfolgt in analoger Weise. Durch Vergleich gleichgrosser Substanzmengen an den korrespondierenden Stellen der vier Teilchromatogramme konnte eine sehr gute Reproduzierbarkeit der Farbbildung festgestellt werden. Während die Leerwerte des Papiers an verschiedenen Stellen *eines* Teilchromatogrammes recht charakteristisch verschieden sind, stimmen sie an korrespondierenden Stellen der vier Teilchromatogramme gut überein. Die Genauigkeit der Methode hängt natürlich davon ab, in welcher relativen Konzentration die zu bestimmende Aminosäure im Gemisch vorliegt. Unter Optimalbedingungen wurden bei jeweils vier Einzelbestimmungen Maximalabweichungen von etwa 5 % gefunden.

Das Verfahren hat sich in umfangreichen Serienuntersuchungen bewährt. Eine genaue Beschreibung der Methode, sowie Angaben über die Reproduzierbarkeit und über ein geeignetes Laufmittelsystem sollen einer grösseren Arbeit vorbehalten bleiben.

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Detection of chitin oligosaccharides on paper chromatograms

The chromatography of oligosaccharides in general has been reviewed by BAILEY AND PRIDHAM[1], and BARKER *et al.*[2] have presented some data on chromatography of chitin oligosaccharides using a pentan-2-ol–pyridine–water mixture followed by detection with alkaline silver nitrate. Many of the reagents which have been described for the detection of sugars on chromatograms depend upon a reaction with

the reducing groups of the sugar. Although these reagents are satisfactory with monosaccharides, the amount of colour obtained with an oligosaccharide series decreases rapidly with the increase of molecular weight. The detection of sugars with silver depends upon a rather generalised reaction, since it can be used for sucrose and trehalose, but it is not satisfactory for oligosaccharides from chitin (Fig. 1). JEANES, WISE AND DIMLER[3] were presumably forced to apply large samples of starch digests to their chromatograms because of the low sensitivity of dinitrosalicylic acid with the oligosaccharides. With a view to increasing the sensitivity and specificity in detection of oligosaccharides in chitin digests, the chlorination reaction of RYDON AND SMITH[4] and the Morgan-Elson reaction as described by SALTON[5] were examined as follows.

All chromatograms were prepared on Whatman No. 1 paper by ascending development with isoamyl alcohol (BDH)–pyridine–water (1:1:0.8). This solvent was found to give better results than pentan-2-ol–pyridine–water (1:1:1) (BARKER

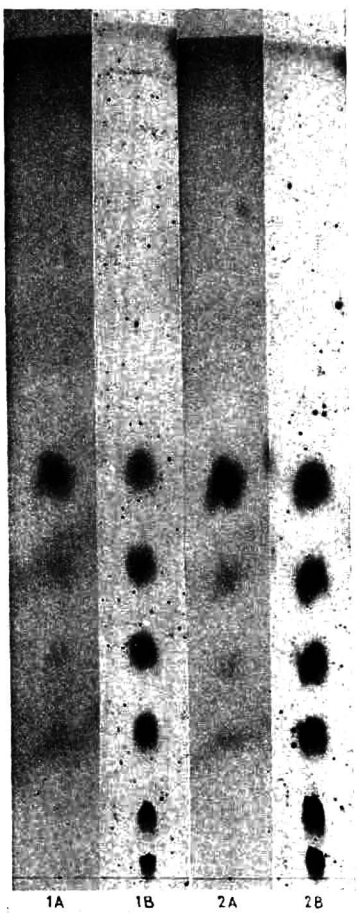

Fig. 1. Chromatograms of chitin oligosaccharides. Solvent: isoamyl alcohol–pyridine–water (1:1:0.8). (A) silver detection; (B) chlorine–potassium iodide–starch detection; (1) 2 μg and (2) 5 μg of saccharide each. The spots are hexasaccharide to acetylglucosamine, in ascending order.

et al.[2]). Chlorine was prepared in a generator from 100 ml concentrated hydrochloric acid and 50 g potassium permanganate, then washed by passing through water and concentrated sulphuric acid and absorbed in 1 l carbon tetrachloride. About 5 g barium carbonate and 5 g anhydrous calcium chloride were added to the flask. Starch-iodide solution was freshly prepared by boiling 1 g starch, 0.25 g potassium iodide and 1 ml 5 *N* HCl in 100 ml water.

The chromatograms were air dried to remove the solvent, then humidified for 2 h in a cylinder with a water-saturated atmosphere. The papers were then rolled and soaked in the chlorine reagent for 20 min. The use of chlorine solution as a dip was more convenient than the use of chlorine gas (RYDON AND SMITH[4]) or spraying techniques (MAZUR *et al.*[6]). After aeration for $^1/_2$ to 1 h to remove the excess chlorine, the papers were sprayed with the starch-iodide reagent. The resulting blue colour in the spots was stable for months and the technique is simpler than that described by BAROLLIER[7], in which the colour is stabilised by treatment with ammonium molybdate. The limit of sensitivity was less than 1 μg and the colour values permitted a roughly quantitative estimation by eye. Fig. 1 illustrates the results using purified chitin oligosaccharides (BARKER *et al.*[2]) and demonstrates the sensitivity of the chlorination method compared with silver treatment.

The Morgan-Elson reaction may be used for detection of acetylamino sugars and also for compounds of acetylglucosamine and acetyl muramic acid in digests of cell walls of microorganisms[5]. The sensitivity of the reaction is very low for chitin oligosaccharides, and DIERICKX AND GHUYSEN[8] found that the limit of detection with the Ehrlich reagent for chitobiose and chitotetraose was 100 μg. This phenomenon was discussed by KUHN *et al.*[9], who showed that 3- or 6-substituted acetylglucosamine compounds give colours with Ehrlich's reagent, but substitution in the 4-position (as in chitin) strongly depresses the colour yield. A simple technique to permit the use of the fairly specific Ehrlich reagent with chitin oligosaccharides is to digest the oligosaccharide spots on the chromatogram with a crude chitinase which produces acetylglucosamine. Chromatograms were lightly sprayed with a solution of a freeze-dried preparation from the puff-ball *Lycoperdon perlatum*[10] (2 mg/ml in 0.05 *M* citrate buffer, pH 4.5), then held in a moist atmosphere for 1 h before applying the SALTON[5] method. Under these conditions good spots with similar colour densities per unit weight of oligosaccharide were obtained.

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Separation of the radiolysis products of o-iodobenzoic acid

The expected products of radiolysis of *o*-iodobenzoic acid are benzoic acid, mono- and di-hydroxybenzoic acids and diphenic acid. For the study of the radiation chemistry of *o*-iodobenzoic acid the separation and quantitative determination of all these aromatic acids were required.

Many authors[1–3] have studied the chromatographic behaviour of mono- and di-hydroxybenzoic acids in different solvents and have determined the respective R_F values, but no method has been reported which yields a clean separation of all the acids we were concerned with and which would permit their subsequent quantitative determination.

A method has been developed, which permits the separation and semi-quantitative determination of all these acids by means of ascending chromatography, using a single solvent consisting of benzene–propionic acid–water (2:2:1)[3]. The acids were detected by four different methods: spraying; ultraviolet illumination; in the case of benzoic acid, which does not react with the developers used in this solvent, detection was performed by automatic scanning or by autoradiography, in comparison with ^{14}C-labelled standards; diphenic acid was detected by two-dimensional chromatography using two different solvents (see Table I).

Experimental

Samples containing 10 λ of 10^{-3} M solutions of the acids were spotted on Whatman No. 1 chromatography paper. After a run of 4–5 h, during which time the solvent

TABLE I

SUMMARY OF R_F VALUES OBTAINED FOR THE DIFFERENT ACIDS AND OF THE METHODS OF DETECTION

Acid	R_F	*Method of detection*
Benzoic	0.98	Scanning; autoradiography
Salicylic	0.93	U.V., spraying with diazotised *p*-nitroaniline (DPNA) + K_2CO_3 solution[4]
m-Hydroxybenzoic	0.77	U.V., DPNA spray
p-Hydroxybenzoic	0.83	U.V., DPNA spray
2,3-Dihydroxybenzoic	0.55	U.V., DPNA spray
2,4-Dihydroxybenzoic	0.40	U.V., DPNA spray
2,6-Dihydroxybenzoic	0.15	DPNA spray
3,4-Dihydroxybenzoic	0.25	Ammoniacal silver nitrate spray[4]
3,5-Dihydroxybenzoic	0.11	DPNA spray
Diphenic	0.06	Two-dimensional chromatography using first the propionic acid–benzene–water system, then the paper is rotated 90°, introduced into butanol–benzene–ammonia buffer (80:5:15)[5] solvent and sprayed with alcoholic methyl red in phosphate buffer[5]

travelled a distance of about 30 cm, the paper was dried and the acids detected by the above-mentioned methods.

The R_F values are averages for the results obtained in 10 determinations. The relative standard deviation calculated from 10 determinations was 3.0 %.

Applications

As seen from Table I the differences between the R_F values of the individual acids are large enough to permit identification on the same paper strip. If quantitative determination is required, the strips may be cut at the respective R_F values, the acids eluted and their U.V. absorbance determined with a spectrophotometer.

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Chromatography on ion exchange papers

XVII. The adsorption of metal ions on cation and anion resin papers from nitric acid

The adsorption of metal ions on cation exchangers from hydrochloric acid, perchloric acid and sodium perchlorate was studied by column methods[1] as well as ion exchange papers techniques[2,3], and it was observed that above a certain concentration of acid (or salt) the adsorption of metal ions again increases.

As no data for the adsorption of metal ions from nitric acid were available the present study was undertaken. It was further considered of interest to compare cation and anion resin papers for a wider range of ions than studied previously[4].

The technique used here was identical to that used in previous studies[2–4], the nitrate form of the anion resin paper (SB-2) being prepared by equilibrating the chloride form with an ammonium nitrate solution. The cation (SA-2) resin paper was employed in the hydrogen form. When the metal ions were spotted on the dry anion resin paper and developed with HNO_3, some ions (notably the rare earths) were prone to double spotting, one spot moving with the liquid front. This could be avoided by running the developing solvent for 3 cm over the paper and spotting the metal ions behind the liquid front.

The results obtained are shown in Fig. 1 where the metal ions are arranged in the order of the periodic table. The following points of interest were noted:

(1) There is an increase in adsorption above 6 N HNO_3 on the cation resin paper of the same magnitude as was observed in perchloric and hydrochloric acids.

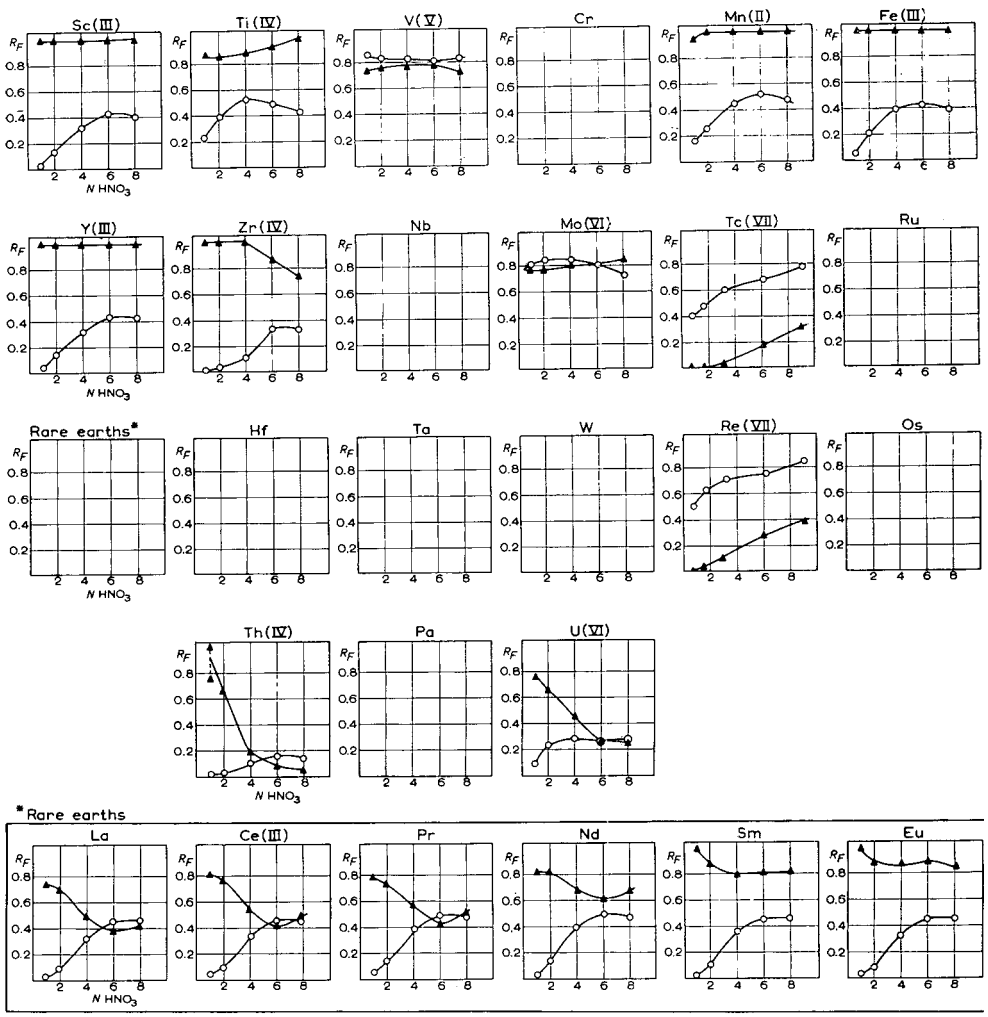
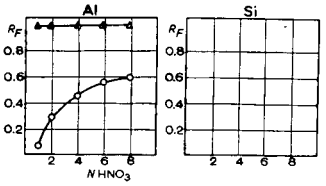
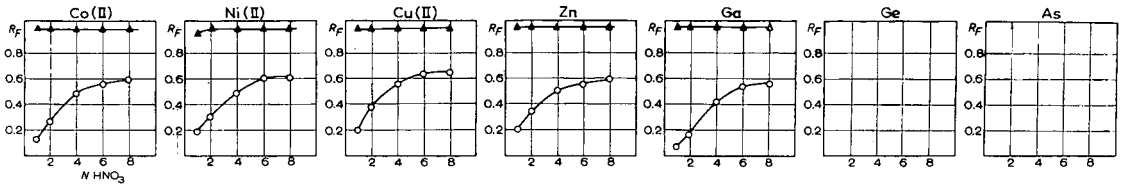
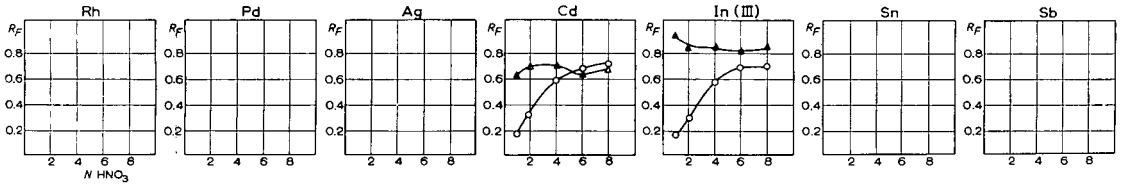
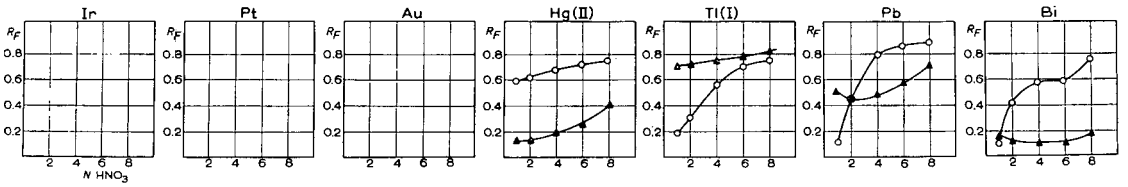


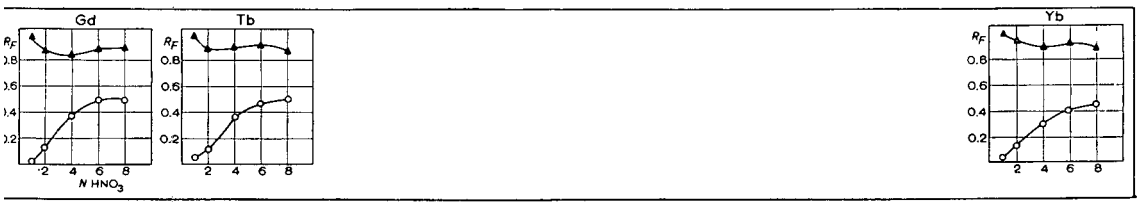
Fig. 1. R_F values of metal ions on cation (SA-2) and anion (SB-2) resin papers with nitric acid as solvent. (⊙) cation (SA-2) paper; (▲) anion (SB-2)paper.


Al
Si
R_F
0.8
0.6
0.4
0.2
2 4 6 8
N HNO3


Co(II)
Ni(II)
Cu(II)
Zn
Ga
Ge
As
R_F
0.8
0.6
0.4
0.2
2 4 6 8
N HNO3


Rh
Pd
Ag
Cd
In (III)
Sn
Sb
R_F
0.8
0.6
0.4
0.2
2 4 6 8
N HNO3


Ir
Pt
Au
Hg(II)
Tl(I)
Pb
Bi
R_F
0.8
0.6
0.4
0.2
2 4 6 8
N HNO3


Gd
Tb
Yb
R_F
0.8
0.6
0.4
0.2
2 4 6 8
N HNO3

(2) All the rare earths have almost identical R_F curves on the cation resin paper in spite of the fact that the early rare earths yield neutral and anionic complexes as is seen from the R_F curves on the anion resin paper.

(3) The anion exchange behaviour here observed confirms essentially the data reported by FARIS AND BUCHANAN[5] with column experiments.

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J. Chromatog., 17 (1965) 625–628

Preparative separation of allergenic macromolecular compounds from airborne particles using continuous electrophoresis*

Previous studies conducted by this laboratory accomplished the separation of minute amounts of two biologically active macromolecular compounds from samples of airborne particles. Paper electrophoresis and paper chromatography were used to separate the two compounds from the samples of heterogeneous material[1,2].

This report describes a method for preparing pure fractions of these compounds in quantity with the aid of continuous-flow curtain electrophoresis. With this method it was possible to accumulate a sufficient amount for biological experimentation even though the compounds were present in trace amounts. An important factor in this procedure was the necessity of maintaining the basic nature of the original compounds.

The method was developed around the use of a Beckman Spinco model CP continuous-flow curtain electrophoresis unit. The material to be separated was prepared in the same way as for paper electrophoresis. Approximately 500 mg of a sample of airborne particles, collected on a fiber glass filter were extracted with 100 ml of 65 % ethanol using a mechanical stirrer at 26° for 8 h[2]. After centrifugation, the clear supernatant was concentrated *in vacuo* to 25 ml. To prevent denaturation of the organic protein materials it was necessary to perform the concentration of the sample at 4°.

The prepared extract was applied to the top of the lower curtain at a predetermined place according to the magnitude of the deflection in a fine stream. For best results the material was applied at the rate of 0.1 ml/h. Barbital buffer, pH 8.6 ionic

* This work was performed under Research Grant Number APoo090-06 from the Division of Air Pollution, Bureau of State Services, Public Health Service.

strength 0.025, was used as an electrolyte. The instrument was operated with Constat power supply at 600 V and at room temperature (26°). Heat produced in the curtain by the passage of current must be drawn off, otherwise evaporation of electrolyte fluid on the curtain will produce a concentration change and alter the ionic strength of electrolyte. To prevent this and to maintain equilibrium conditions, the cell was sealed against vapor loss. Balanced electrolyte pump and overflow systems maintain an even flow of buffer down the curtain. In addition, temperature control of the cell was necessary to protect the labile material[3]. It was found experimentally that the allergenic activity of the desired compounds was preserved if the cell temperature was kept at 23° throughout the separation. To accomplish this the cooling plate behind the curtain was connected to a closed circulating system in which a 20% ethylene glycol solution was passed through a coil immersed in a cooling bath kept at 6°. This system maintained the temperature inside the cell at 23°. Readings were taken every 10 min throughout the run to insure proper operation. After an experimental run of 24 h the curtain was dried as quickly as possible with circulating warm air.

In the separation of the extracted material two migration paths A and C were clearly visible from the natural pigments without any chemical treatment (Fig. 1). Treatment of the curtain with appropriate chemical reagents revealed the location of the other separated compounds on the curtain and in the same time determined which of the collecting tubes contained the separated substances. Upon spraying the curtain with aniline oxalate reagent (100 ml of 0.1 *N* oxalic acid plus 0.9 ml of aniline) and heating to 100° for 15 min the path of compound B was revealed (Fig. 1).

To locate the flavonoid containing compounds the curtain was sprayed with

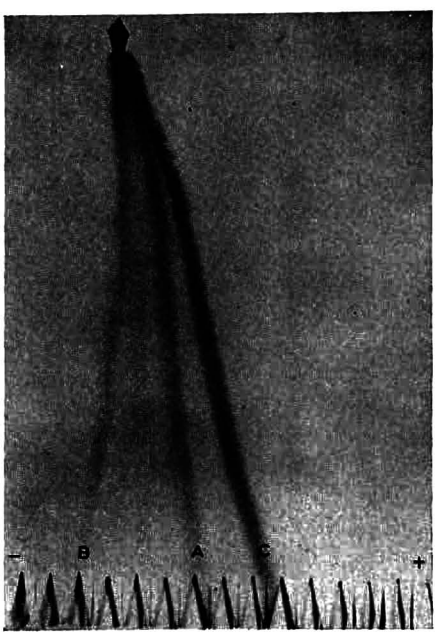

Fig. 1. Continuous electrophoretic separation of allergenic macromolecular compounds from extract of airborne particles.

bis-diazotized benzidine and the path of compound A was revealed. It corresponded to the location of one of the visible yellow lines on the dried curtain.

Solutions of the separated substances in the buffer were transferred to a standard Visking tubing and dialysed against 10 l of demineralized water for 2 h at 23° to remove the buffer. In order to get more efficient action in a shorter period of time, the Visking tubing with the material was slowly rotated in the demineralized water. After 2 h the dialysed material was concentrated *in vacuo* to 1 ml and transferred to a sterile vial. The separated compounds were then used in skin testing to determine their allergenic nature. To prove the compounds' identity and uniformity the isolated fractions were tested with paper electrophoresis on a Reco model E 800-2 unit using barbiturate electrolyte at pH 8.6 ionic strength 0.05 and with operating conditions as reported in an earlier work[2]. Each compound formed a single line in a previously determined position indicating the purity of the material. Upon examination in ultraviolet light similar characteristics of the compounds, as observed in earlier work, were evident.

Discussion

In working with allergenic compounds of high molecular weight, it is extremely important to define and control the experimental conditions so that compound degradation and consequent loss of allergenic activity does not occur.

One of the most important factors found in this work was temperature. It is possible to operate the entire CP cell in an accessory refrigerator. The advantage of the refrigerator is that greater field intensities can be obtained which would increase the resolution of thermolabile substances and, in addition, provide a proper control of temperature. However, negative results were obtained using a refrigerator due to temperature differences throughout the cell and curtains. It was found experimentally that the best results were obtained in operating the unit at room temperature and circulating a cool liquid through the cooling plate behind the curtain and not using the refrigeration system. The temperature on the curtain was kept at 23° by means of the circulating liquid cooled to 6°.

A small variation in temperature gave separation pattern changes in a short time and resulted in contamination of the separated substances in the collecting tubes.

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Determination of auric ions in colloidal gold-198 by paper electrophoresis

The determination of auric ions in colloidal gold-198 preparations has so far been undertaken by means of paper chromatography. We have found it desirable, however, to verify the analytical results obtained by the various solvents for chromatography, and in this connection a quantitative method utilizing paper electrophoresis has been worked out.

Reviewing earlier work, MUKERJEE[1] has qualitatively examined the electromigration of auric ions in various electrolytes. A running period of 12 h is used in this procedure, and DE VRIES *et al.*[2] recently published a qualitative method of analysis for mixtures of metallic ions including auric ions by paper electrophoresis. Citric acid is used as supporting electrolyte. LEDERER[3] also reports the use of paper electrophoresis by 0.1 *N* hydrochloric acid for quantitative separation of copper from gold. Some other mediums have been utilized for the separation of gold from other precious metals[4].

We have been searching for a method by which the auric ions separate rapidly from the colloidal gold particles, without tailing. In addition, emphasis has been laid on obtaining a straightforward procedure requiring a simple and inexpensive apparatus. Finally, the electrolyte should be indifferent in the face of the colloidal system and keep the auric ions in the anionic complex state, which, according to some authors[5,6], will give a good separation.

In introductory experiments the following electrolytes were tried: Borate buffer, citrate buffer (both at various pH's), sodium chloride, sodium thiosulphate (both at various pH's), acetic acid, hydrochloric acid and sodium hydroxide. All the electrolytes were tried at various concentrations. With sodium chloride, hydrochloric acid and sodium hydroxide the auric ions did not separate satisfactorily, but were found to spread in one or both directions from the application point without forming one definite zone. Potassium chloride and some other mediums have previously been found to give the same effect[1]. Acetic acid, borate buffer and citrate buffer did not separate the auric ions from the colloidal gold, 0.075 *M* sodium thiosulphate gave a rapid separation and was finally chosen as supporting medium. In this medium auric ions migrate towards the anode as expected[1].

Experimental

A horizontal electrophoresis apparatus (2549 Shandon Universal Electrophoresis Apparatus, after Kohn) was used in connection with a constant voltage/current pack. Whatman paper No. 1 and cellulose acetate paper were tried. However, since the latter seemingly did not exhibit any advantage to the former, Whatman paper No. 1 was used in subsequent work. Paper strips (18 × 2 cm) were wetted in the supporting medium, followed by clamping between filter papers. 5 μl of the samples under investigation were applied as a streak in the middle of the strips. By using constant current (2.5 mA/cm width of paper) auric ions were found to migrate approximately 65 mm towards the anode after one hour's run. The potential usually dropped from 400 to 200 volts. Colloidal gold particles moved 2–5 mm towards the anode under the same experimental conditions. All our experiments were performed using radioactive gold-198, consequently no developing reagent was necessary. The radioelectrophoretograms were scanned by means of the Frieseke-Hoepfner FH 452 automatic scanner.

Results

Fig. 1 shows a typical scan of a made-up sample of colloidal gold, showing an ionic gold content of 28 %.

The accuracy of the method as established by comparison with paper chromatography[7] corresponds to a deviation of 30 % or less in the lower ionic region (1–2 % ionic gold), which usually is the region of main interest. The accuracy was far better in the higher ionic region. The precision has been found to be better than 10 %.

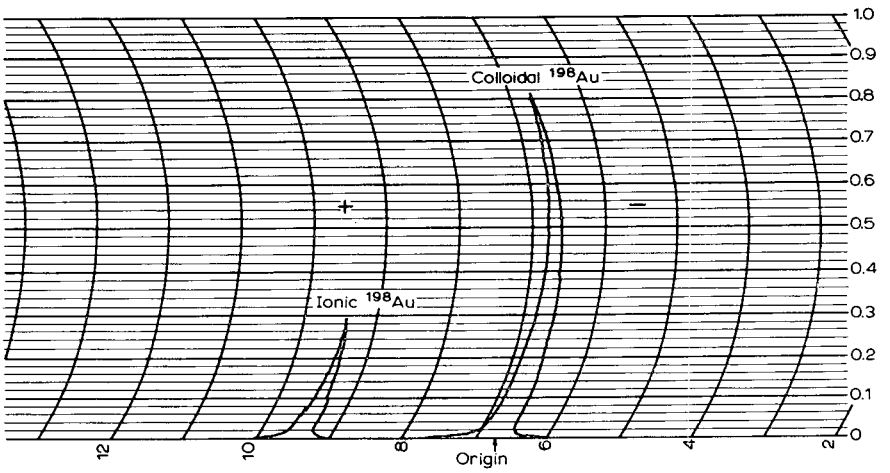

Fig. 1. Scannogram of colloidal ^{198}Au and ionic ^{198}Au.

Special attention has been paid to the possibility that sodium thiosulphate might influence the colloidal system, *i.e.* reduce auric ions or otherwise interfere. Experiments have shown, however, that 0.075 M sodium thiosulphate does not change the percentage of auric ions in colloidal gold-198 preparations.

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U. BEEN
A. HØYE

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Book Reviews

Dünnschicht-Chromatographie mit Adsorptionsmitteln Woelm (Woelm-Mitteilungen AL 10), M. Woelm, Eschwege, 1963.

It has become common place nowadays for manufacturers of chromatographic material and equipment to publish smaller or larger monographs explaining the applications of their products. Often these constitute also a useful introduction to a particular technique, as for example the booklet under discussion which is a concise 30-page introductory account of thin-layer chromatography. Its only shortcoming is its limitation to adsorbents (*i.e.* Woelm adsorbents) and a complete absence of any mention of cellulose thin layers. Nevertheless it can be recommended for beginners, both students and chemists who want to learn about thin-layer chromatography without having to read an extensive treatise.

The booklet is well-illustrated and appears not only in German but in English as well.

J. Chromatog., 17 (1965) 633

Separation Methods in Biochemistry, by C. J. O. R. MORRIS AND P. MORRIS, Pitman, London, 1964, 887 pp., price £ 5.15.0.

This book is based on a series of lectures given by one of the authors at University College, London, and all the separation methods used in biochemistry *viz.* chromatography, electrophoresis, countercurrent distribution, membrane separation methods, etc. are discussed. It is the kind of book which is invaluable for a university course, both for preparing lectures and for selecting reading matter for students. The treatment of the principles underlying the separation methods is also at a level which makes it suitable for the research worker, while applications to specific problems are only used for illustrative purposes and are not comprehensive.

While most sections are well compiled and clearly written, one feels that there is a certain lack of balance in some of the more important fields, for example, gas chromatography and thin-layer chromatography receive rather little attention in relation to their importance. The reason for this is evident from the reference lists. It seems that papers later than 1959 were inserted as an afterthought and have a, b, c, etc. numbers, but even amongst these there are few later than 1961. We have thus an account prepared five years ago and brought up to date three years ago with further additions during the printing. Given the rapidly developing field covered and the tremendous advances made in some of these fields during the recent years one symphathises with the authors and hopes that the success, which the book should have, will induce them to prepare an up-to-date edition in the near future.

J. Chromatog., 17 (1965) 633

Protides of the Biological Fluids, Proceedings of the Eleventh Colloquium, Bruges, 1963, edited by H. PEETERS, Elsevier Publ. Co., Amsterdam-London-New York, 1964, 536 pp., price Dfl. 70.—, £ 7.0.0, DM 78.—, clothbound.

It is unnecessary to repeat the enthusiastic remarks with which the previous volumes of this regular series have been greeted in this journal. The readiness with which the Bruges colloquia reflect basic trends and recent fashions makes these collections of papers a sensitive indicator of the present-day effort in the field of protein research, in so far as it is directly or indirectly related to medical problems. The number of papers (113 communications, reviews and experimental papers, and 3 round-table discussions) would almost seem to justify an attempt at statistical evaluation which, of course, would have to be interpreted with caution.

The Colloquium was divided into 4 sections: Section A (A stands for "academic") on *structure and immunology of proteins* with 33 papers and a round-table discussion, Section B on *protein-losing diseases* with 22 papers and a round-table discussion, Section C on *glyco- and mucoproteins* (including glycopeptides) with 13 papers, Section D on *techniques* with 31 papers and a round-table discussion, and Section E on *various topics* (mainly isoenzymes) with 14 papers. The geographical distribution of the laboratories from which the papers originated may give some information on the force of attraction which the annual Bruges pilgrimage exerts on the protein scientists from different parts of the world. Most papers were in English (69), but there were 26 in French (F) and 18 in German (G); the numbers of papers in the latter two languages will be given in brackets. From the Americas and Africa the distribution was as follows: U.S.A. 19, Canada 2 (1 F), Brazil 1, Senegal 1 (1 F), Congo (Leopoldville) 1 (1 F), while the rest were from Europe: France 17 (16 F), Great Britain 14, Germany 14 (13 G), Belgium 13 (5 F), Denmark 7, Sweden 7, Italy 5, Holland 4, Switzerland 4 (2 F, 2 G), Austria 2 (2 G), Bulgaria 1 (1 G), Finland 1.

Table I shows the popularity of various chromatographic and electrophoretic techniques among the participants. Since several techniques were often used in one

TABLE I

POPULARITY OF CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES AMONG PARTICIPANTS*

	Chromatography (16) *and Chromatography + electrophoresis* (9)		*Electrophoresis* (50) *and Chromatography + electrophoresis* (9)	
No. of papers	25		59	
Methods used	Gel filtration (Sephadex)	(15)	Paper	(10)
	Ion exchange (cellulose)	(12)	Cellulose acetate	(2)
	Ion exchange (Sephadex)	(1)	Starch gel	(11)
	Ion exchange (resin)	(2)	Starch gel immuno-	(1)
	Paper	(5)	Agar	(17)
			Agar immuno-	(25)
			Agarose immuno-	(1)
			Polyvinyl	(4)
			Polyacrylamide	(3)
			Density gradient	(1)

* Number of papers in parentheses.

study, simple sums of the figures would naturally exceed the total of the papers.

It can be seen that chromatographic and/or electrophoretic techniques were used in 75 *i.e.* two thirds of all the papers. In a few cases the choice of a certain technique might have been arbitrary, but mostly a specific purpose was served by a particular technique.

The round-table discussions merit special comment. It is well known how difficult it is to give to a short period of discussion the appearance of informality without losing brevity and coherence; the more un-edited it has to look, the more editorial work does it require. Dr. PEETERS has mastered this part. Even the mysterious "doctor from the floor" and "voices" serve to create the atmosphere. The chairmen made the best of the limited time available in channelling the discussion to those topics around which most argument was likely to arise. J. F. HEREMANS piloted the immunological discussion to cover both the non-γ-components as antigens and γ-globulins as antigens and antibodies. One sees how the classification of the γ-family of proteins is beginning to take shape, accounting for their characteristic differences and similarities. Unfortunately, no agreement was reached on the nomenclature of the main groups. The picture of the chemical structure of the γ-components was lucidly explained by R. R. PORTER. The basic pattern includes 2 lighter and 2 heavier polypeptide chains connected by disulphide bridges and exhibits a *locus minoris resistentiae* within the heavier chains. Other authors shared these views. The brilliant introductory lecture in which P. GRABAR presented a new conception of antibodies as "globulines-transportateurs", thus obviating some difficulties of interpretation and creating some new ones, was not touched on.

Digestive protein loss has long been neglected, but now rightly comes into focus. P. VESIN, as the chairman of the round-table discussion on this subject, made an effort to show the shortcomings of the present state of knowledge. Thus, for example, the plasma albumin normally catabolized in the gastrointestinal tract may, according to the methods and assumptions employed, be assessed as being between 10 and 66% of the total, and this is the background against which the protein-losing gastroenteropathy has to be diagnosed and evaluated.

The round-table discussion on techniques included about 4 chromatographic and 10 electrophoretic topics. The transfer method of KOHN ("father of the membranes"), which was used in conjunction with cellulose acetate strips and was only described in the discussion, seems to promise an improved resolution of the immuno-electrophoretic patterns. Those interested in the relative merits of various kinds of ion-exchange polysaccacharides may find the experimental paper by C.S. KNIGHT useful in helping to provide a rational background for their choice. Instances in which ion-exchange cross-linked dextrans were superior, were mentioned in the discussion. A. H. SEHON has drawn attention to fully synthetic molecular sieves based on hydrophilic polymers, *e.g.* of acrylamide type. His question as to the obvious advantages of stepwise and gradient elution was answered by quoting SOBER's answer that he liked both of them and that ion exchange was not yet a science in its own right.

No revolutionary technical developments appeared in the Colloquium; but the commercial automatic density gradient electrophoresis apparatus demonstrated by H. LERNER, or the direct nephelometry of protein precipitates in agar electrophoresis (R. LONTIE, *et al.*) are worth mentioning.

It need not be emphasized that the paper and printing are of the usual high

standard, but frequent printer's errors and mis-spelling are again the price paid for promptness in publication.

In some papers the names of the authors follow the head-lines, whereas in others they appear at the end; the reason for this difference is not obvious.

I. M. HAIS (Hradec Králové)

J. Chromatog., 17 (1965) 634–636

Announcement

JOURNÉES INTERNATIONALES DE LA SÉPARATION IMMÉDIATE ET DE LA CHROMATOGRAPHIE

The Greek Chemists' Association, in cooperation with the Association for the Development of Spectrographic Methods (G.A.M.S.—France), is organizing the 3rd International Meeting under the above title.

This meeting will be held in Athens on September 19th–25th, 1965.

For further information apply to the Chairman of the Meeting:

Dr. G. PARISSAKIS, Professor of the Technical University of Athens, Patission 42, Athens, Greece;

or to:

G.A.M.S., 1, Rue Gaston Boissier, Paris 15e, France.

J. Chromatog., 17 (1965) 636

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See also PC section.

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3. TECHNIQUES I

3a. Detectors

EADS, E. A. AND PAYNE, JR., D. A.: Catalyzed copper–copper oxide flame reactions of selected organohalogens in the presence of methane and air. *Anal. Chem.*, 36 (1964) 2373 — selective test for halogens, B,N,P and S.

KAYE, W. AND WASKA, F.: Å rapid-scan far ultraviolet spectrophotometer for monitoring gas chromatograph effluent. *Anal. Chem.*, 36 (1964) 2380–2381 — 1600–2100 Å in 6 sec (resolution > 1 Å); a special wavelength drive and synchronized gain circuit compensates for background energy variation with wavelength.

MCFADDEN, W. H. AND DAY, E. A.: Scan rate consideration in combined gas chromatography–mass spectrometry. *Anal. Chem.*, 36 (1964) 2362–2363 — for most cases scan rates of 1–4 sec and mass resolution of 300 are adequate.

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CHOVIN, P.: Les phases utilisées en chromatographie en phase gazeuse. Mode d'action, classement, emploi. *Bull. Soc. Chim. France*, (1964) 1800–1810 — classification of and many data for cyano-ethoxy derivatives.

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URBACH, G.: Ion exchangers for gas solid chromatography. *Anal. Chem.*, 36 (1964) 2368 — Dowex 50-8X (in acid form) with 1.5% squalane gives symmetrical peaks for C_6–C_9 fatty acids.

VERGNAUD, J. M., DEGEORGES, E. AND NORMAND, J.: Progrès dans l'analyse chromatographie gazeuse: chromatographie avec inversion des gaz (backflushing). *Bull. Soc. Chim. France*, (1964) 1904–1910 — theory and apparatus are given.

ZLATKIS, A., CHAD, G. S. AND KAUFMAN, H. R.: Substrates containing silver nitrate for gas chromatography. *Anal. Chem.*, 36 (1964) 2354 — for room temperature *p*-xylyl cyanide, for a higher temperature 1-naphthylacetonitrile, 3,4-dimethylphenylacetonitrile and oleyl nitrile are described as sufficiently stable stationary phase carriers.

3c. Apparatus, accessories and materials for GC

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HAMILTON, L. H.: Two-loop sampling valve for use in gas chromatography. *J. Gas Chromatog.*, 2 (1964) 302–303 — construction details are given.

KELLEY, T. F.: Simple variable-temperature sample inlet for Beckman GC-2 gas chromatograph. *Anal. Chem.*, 36 (1964) 2374 — applied for C_{14}–C_{18} fatty acid esters.

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4a. Preparative-scale GC

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4d. Special microtechniques

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YANOVSKIĬ, M. I. AND GAZIEV, G. A.: (Impuls-frontal gas–liquid chromatography). In ZHUKHOVITSKIĬ, A. A. *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 78–83 — reading from sorption and desorption part of chromatogram possible; useful for small concentrations.

4f. Measurement of physico-chemical and related values

KNÖZINGER, H. AND SPANNHEIMER, H.: Zur Bestimmung von Adsorptionsenthalpien mit Hilfe der Gaschromatographie. *J. Chromatog.*, 16 (1964) 1–6 — measured with benzene; at a surface coverage of $5 \cdot 10^{-7}$ mol per g of silica gel and at temperatures from 200° to 320° values of 8–11 kcal/mol were found with 5% deviation between the measurements.

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5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

BARANOVA, V. G., PANKOV, A. G., KHRININ, E. G., GLAZYNINA, P. V., BELYAEVA, V. D., OBESHCHALOVA, N. V., DOLGOVA, N. A., KNYAZEVA, M. F., MISHINA, A. B. AND IVOYLOVA, M. A.: (Application of gas chromatography in the production of monomers for synthetic caoutchouc). In ZHUKHOVITSKIĬ, A. A. *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 109–115 — analysis of products of butadiene synthesis.

BRUK, A. I., VINOGRADOVA, L. M. AND VYAKHIREV, D. A.: (Investigation of conditions for chromatographic separation of gas mixtures in the automatic control of a butene dehydrogenation process). In ZHUKHOVITSKIĬ, A. A. *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 119–124.

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5b. Other hydrocarbon types

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OUCHI, K. AND IMUTA, K.: The analysis of benzene extracts of Yuabari coal. II. Analysis by gas chromatography. *Fuel*, 42 (1963) 445–456 — aromatics from naphthalene to picene on high-vacuum silicone grease at 180°, 250°, 280° and 300°.

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5c. Halogen derivatives of hydrocarbons

CLEMONS, C. A. AND ALTSHULLER, A. P.: A quantitative study of electron capture detector parameters: The determination of halogenated trace substances in air. *148th National Meeting, Am. Chem. Soc., Chicago, Ill., August 31–September 4, 1964. Div. Water, Air, Waste Chem., Abstr. Papers*, p. 8Y, Abstr. No. 19 — SF_6 and $CBrF_3$ up to 10^{-5}–10^{-6} p.p.m.

PROFFT, E. AND OBERENDER, H.: Synthesen mit Methallylchlorid (= 1-Chlor-2-methylpropen-(2)). *J. Prakt. Chem.*, [4] 25 (1964) 225–271 — C_4 halogen derivatives on PEG at 140°.

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HARTMAN, L.: Estimation of glycerol and diglycerol in the presence of each other. *J. Chromatog.*, 16 (1964) 223–225 — retention data as acetates on silicone grease and SE-30 at 207°.

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STEINKE, R. D.: Phenols from grain, the production of steam-volatile phenols during the cooking and alcoholic fermentation of grain. *J. Agr. Food Chem.*, 12 (1964) 381–387 — retention data on DC-200 and Carbowax 1500 at 160°.

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GOLDFINE, H.: Composition of the aldehydes of *Clostridium butyricum* plasmalogens. Cyclopropane aldehydes. *J. Biol. Chem.*, 239 (1964) 2130–2134 — retention data of aldehydes as dimethylacetals on PEGA, Apiezon M and SE-30 at 185°, 197° and 185°.

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10. CARBOHYDRATES

MAKITA, A. AND YAMAKAWA, T.: The glycolipids of the brain in Tay-Sach's diseases. The chemical structures of a globoside and main ganglioside. *Japan J. Expl. Med.*, 33 (1963) 361–368 — methyl glycosides of methylated sugars on neopentyl GS at 198°.

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11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

BYARS, B. AND JORDAN, G.: An efficient packed column for free fatty acids. *J. Gas Chromatog.*, 2 (1964) 304–305 — C_1–C_{18} fatty acids on Carbowax 20M–terephthalic acid polyester by PTGC 170–270° in 9 min and at 270° isothermally.

JOHNSTON, P. V. AND ROOTS, B. I.: A source of contamination in the ultramicro analysis of methyl esters of fatty acids by gas–liquid chromatography. *J. Lipid Res.*, 5 (1964) 477–478.

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KORN, E. D.: The fatty acids of *Euglena gracilis*. *J. Lipid Res.*, 5 (1964) 352–362 — retention data for 51 fatty acids on PEGS, PEGA and Carbowax 400 at 175–190°, 100° and 85°.

NIKELLY, J. G.: Gas chromatography of free fatty acids. *Anal. Chem.*, 36 (1964) 2244–2248 — C_{12}–C_{18} acids on low-coated glass microbeads (200 μ) on DEGS and Carbowax 20M, both with isophthalic acid at 175° and 180°; C_1–C_{11} acids by PTGC 90–175°, 3.3°/min.

11b. Lipids and their constituents

ARRIGO, L. AND TISCORNIA, E.: Gas chromatographic determination of the fatty acids of depot, parenchymal and faecal lipids. Behaviour following partial hepatectomy. *Ital. J. Biochem.*, 12 (1963) 339–354 — on PEGS at 210°.

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KATES, M., ADAMS, G. A. AND MARTIN, S. M.: Lipids of *Serratia marcensens*. *Can. J. Biochem.*, 42 (1964) 461–479 — on butanediol succinate polyester at 180°.

LUNA, G., D'ARRIGO, G., SAGLIMBENE, F. AND TAMBURINO, G.: (Contribution to the study of plasma fatty acids in atherosclerotic patients. An investigation using gas chromatography). *Boll. Soc. Ital. Biol. Sper.*, 39 (1963) 1735–1737 — on PEGS at 210°.

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13. STEROIDS

COX, R. I. AND BEDFORD, A. R.: The use of double derivatives in the gas chromatography of urinary estrogens. *Steroids*, 3 (1964) 663–669 — retention data on GF-1, NGA at 225–250°.

15. TERPENE DERIVATIVES

ICONOMOU, N., VALKANAS, G. AND BÜCHI, J.: Composition of gum turpentines of *Pinus halepensis* and *Pinus brutia* grown in Greece. *J. Chromatog.*, 16 (1964) 29–33 — retention data on PEG 1500 and diisodecyl phthalate at 100° and 102°.

16. NITRO AND NITROSO COMPOUNDS

PHILLIPS, L. V. AND COYNE, D. M.: A study of isobutylene — nitric oxide reaction products. *J. Org. Chem.*, 29 (1964) 1937–1942 — retention data on DNP at 110–115°.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

CARTONI, G. P. AND DE STEFANO, F.: Determination of amphetamines by gas chromatography. Urinary excretion of amphetamines in rat and man. *Ital. J. Biochem.*, 12 (1963) 296–309 — on Carbowax 1500 at 120°.

18. AMINO ACIDS

LAMKIN, W. M. AND GEHRKE, C. W.: Preparation of *n*-butyl-N-trifluoroacetyl esters of amino acids for gas–liquid chromatography. *148th National Meeting, Am. Chem. Soc., Chicago, Ill., August 31–September 4, 1964. Div. Biol. Chem., Abstr. Papers*, p. 21C, Abstr. No. 33.

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19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

WEYGAND, F., PROX, A., JORGENSEN, E. C., AXÉN, R. AND KIRCHNER, P.: Gaschromatographische Trennung von N-Trifluoracetyldipeptid-methylestern. III. Dipeptide mit trifunktionellen Aminosäuren. *Z. Naturforsch.*, 18b (1963) 93–104.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

GUDZINOWICZ, B. J. AND CLARK, S. J.: The gas chromatographic analysis of low concentrations of barbiturates using an electron affinity detector. *148th National Meeting, Am. Chem. Soc., Chicago, Ill., August 31–September 4, 1964. Div. Anal. Chem., Abstr. Papers*, p. 11B, Abstr. No. 27.

22. ALKALOIDS

VAN BINST, G., DENOLIN-DEWAERSEGGER, L. AND MARTIN, R. H.: Application de la chromatographie en phase gazeuse à l'étude des produits de pyrolyse d'alcaloïdes indoliques. I. Obtention des pyrogrammes. *J. Chromatog.*, 16 (1964) 34–39 — seven-membered or bridged six-membered C-ring alkaloids are characterized by their pyrolytic spectra.

24. OTHER SULPHUR COMPOUNDS

HUGHES, R. B.: Chemical studies on the herring (*Clupea harengus*). IX. Preliminary gaschromatographic study of volatile sulphur compounds produced during the cooking of herring. *J. Sci. Food Agr.*, 15 (1964) 290–292 — $(CH_3)_2S$, CH_3SH and H_2S on tritolyl phosphate.

26. METALLO-ORGANIC COMPOUNDS

GEISSLER, H. AND KRIEGSMANN, H.: Zur Analyse der Butylzinnverbindungen $(n\text{-}C_4H_9)_nSnX_{4-n}$. *Z. Chem.*, 4 (1964) 354–355 — on Ph-Me-silicone oil OE 4011 at 160–180°.

28. ANTIBIOTICS

DUGAN, P. AND LUNDGREN, D.: Microdetection of menadione, coenzyme Q6, and certain related quinoid compounds in biological materials using electron capture-gas chromatography. *Anal. Biochem.*, 8 (1964) 312–318 — DC at 180–205°.

29. INSECTICIDES AND OTHER PESTICIDES

BENFIELD, C. A. AND CHILWELL, E. D.: The determination of some triazine herbicides by gas–liquid chromatography with particular reference to atraton in soil. *Analyst*, 89 (1964) 475–479 — retention data of 9 compounds on PEGA at 140°.

BONELLI, E. J., HARTMANN, H. AND DIMICK, K. P.: Gas chromatography retention times and sensitivity data for insecticides and herbicides. *J. Agr. Food Chem.*, 12 (1964) 333–336 — on QF-1 and Dow-11 at 180°.

BURCHFIELD, H. P., RHODES, J. W. AND WHEELER, R. J.: Simultaneous and selective detection of phosphorus, sulphur and halogen in pesticides by microcoulometric gas chromatography. *148th National Meeting, Am. Chem. Soc., Chicago, Ill., August 31–September 4, 1964. Div. Agr. Food Chem., Abstr. Papers*, p. 23A, Abstr. No. 55 — decomposing in H_2 as carrier gas and in quartz tube at 950° results in hydrocarbons, H_2O, PH_3, H_2S and HX.

COOK, C. E., STANLEY, C. W. AND BARNEY, II, J. E.: Correlation of structure of phosphate pesticides with response in electron affinity detectors. *Anal. Chem.*, 36 (1964) 2354–2358 — electron affinity increases as follows: O:P–O > S:P–O < O:P–S < S:P–S.

DAWSON, J. A., DONEGAN, L. AND THAIN, E. M.: The determination of parathion and related insecticides by gas–liquid chromatography with special reference to fenithion residues in cocoa. *Analyst*, 89 (1964) 495–496 — retention data of 4 herbicides on E-301 + Epikote resin 1001 at 160°, 175° and 200° are given.

FRIEDRICH, K. AND STAMMBACH, K.: Gas chromatographic determination of small vapour pressures. Determination of the vapour pressures of some triazine herbicides. *J. Chromatog.*, 16 (1964) 22–28 — inert gas is passed through a saturation column and the transferred substance is condensed in a cooled GC-column; determination of vapour pressures in the order of 10^{-5}–10^{-6} mm Hg by the internal standard method is possible.

GUTENMANN, W. H. AND LISK, D. J.: Herbicide mechanismus, conversion of 4-(2,4-DB) to 2,4-dichlorophenoxycrotonic acids (2,4-DC) and production of 2,4-D from 2,4-DC in soil. *J. Agr. Food Chem.*, 12 (1964) 322–323 — on silicone grease at 200°.

31. PLASTICS AND THEIR INTERMEDIATES

ALEKSEEVA, A. V., BERMAN, S. S., GOLBERT, K. A., DACKEVICH, A. A., MOSHINSKAYA, M. B. AND FOMINA, A. F.: (Determination of microimpurities in monomers). In ZHUKHOVITSKIĬ, A. A. *et al.* (Editors): *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 99–108.

ARNOLD, P. E.: The application of pyrolysis-gas chromatography to the analysis of polymeric materials. *Proc. Soc., Anal. Chem.*, 1, No. 7 (1964) 79–81 — a discussion follows a comprehensive summary of the paper.

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VOL. 17 (1965)

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SUPPLEMENT TO THE
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EDITORS:

C. G. CASINOVI (Rome, Italy)
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TABLE 1

REMOVAL FROM SOLUTION OF ELEMENTS IN 0.1 *M* TO 14 *M* NITRIC ACID WITH STRONGLY BASIC ANION EXCHANGE RESIN DOWEX 1 × 10, 200–400 MESH (J. P. FARIS AND R. F. BUCHANAN, *Anal. Chem.*, 36 (1964) 1157) (Courtesy of *Analytical Chemistry*)

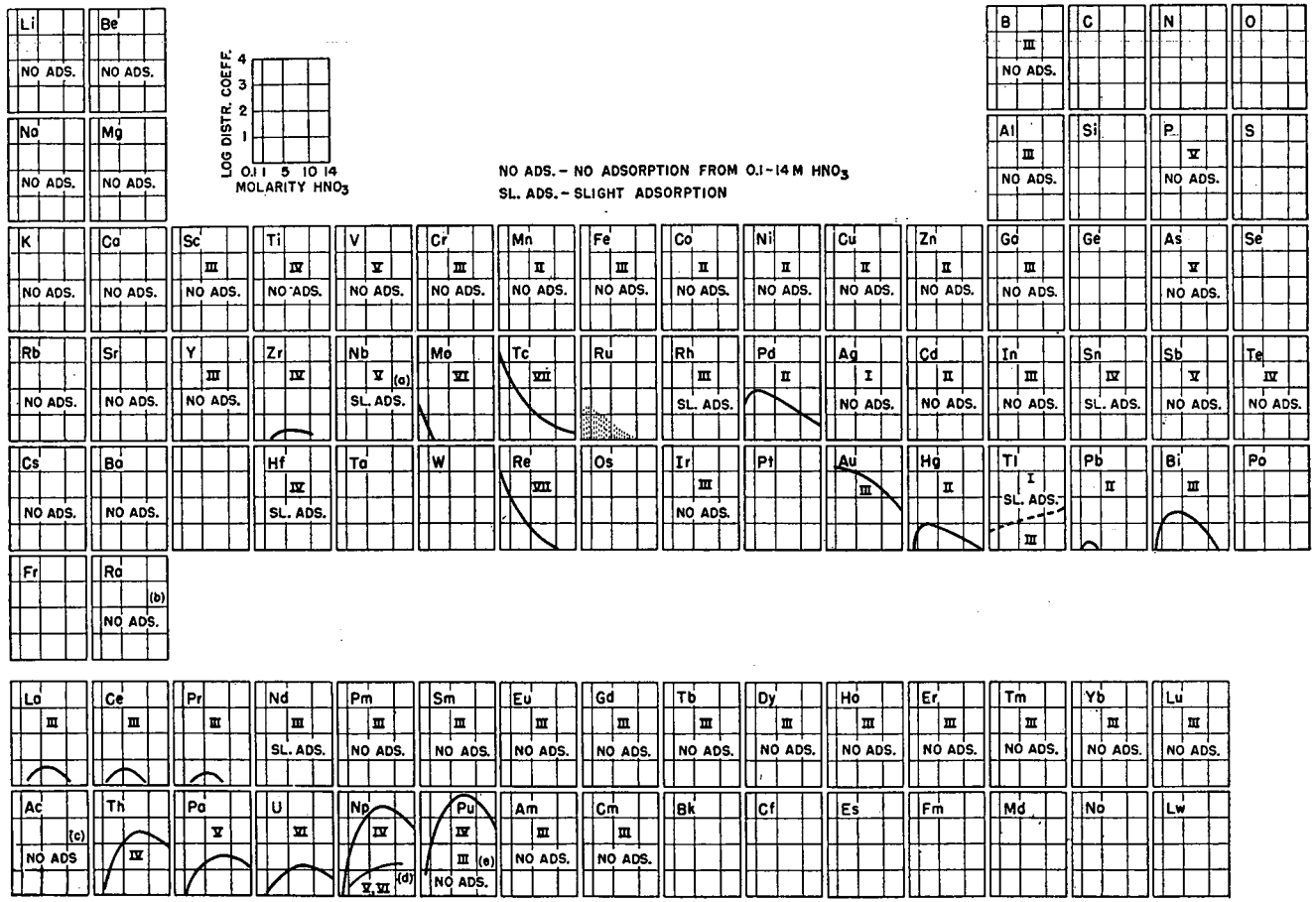


TABLE 2

ELECTROPHORETIC MOBILITIES OF CARBOHYDRATES IN SULPHONATED PHENYLBORONIC ACID BUFFER AS WELL AS GERMANATE AND BORATE BUFFERS

(P. J. GAREGG AND B. LINDBERG, *Acta Chem. Scand.*, 15 (1961) 1913)

Electrolytes: E_1 = pH 6.5 sulphonated phenyl boronic acid buffer.
E_2 = pH 10.7 germanate buffer.
E_3 = pH 10.0 borate buffer.

Paper: Whatman No. 1.

Apparatus: KUNKEL AND TISELIUS type (H. G. KUNKEL AND A. TISELIUS, *J. Gen. Physiol.*, 35 (1951) 89, see also A. B. FOSTER, *Advan. Carbohydrate Chem.*, 12 (1957) 81). Plates cooled by circulating water at 400°).

Potential applied: 500 V (about 10 V/cm). (Note: Buffer in vessels mixed after each 3 h at 500 V.)

Time of run: 3 h or 6 h.

Start marker: Hydroxymethylfurfural.

Zero markers: Glucose or mannitol.

Migration units: M_G = True distance of migration of substance/true distance of migration of glucose.
M_M = True distance of migration of substance/true distance of migration of mannitol.

Detection: D_1 = *p*-Anisidine hydrochloride spray (L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702), for reducing sugars.
D_2 = Periodate–benzidine spray (J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132), for non-reducing sugars and inositols.

Compound	M_G		
	E_1	E_2	E_3
Monosaccharides			
D-Xylose	1.8	1.4	1.00
D-Lyxose	2.3	1.9	0.71
L-Arabinose	2.4	1.5	0.96
D-Ribose	4.7	2.1	0.77
L-Rhamnose	0.50	1.3	0.52
D-Glucose	1	1	1
D-Mannose	1.1	1.4	0.72
D-Galactose	1.8	1.3	0.93
D-Altrose	5.8		0.97
L-Sorbose	8.5	2.0	0.95
D-Tagatose	8.6	2.4	0.95
D-Fructose	9.3	2.1	0.90
Glycosides			
Methyl α-D-xylofuranoside	2.3	0.05	0.33
Methyl β-D-xylopyranoside	0.0	0.0	0.0
Methyl β-D-glucofuranoside	2.0	—	—
Methyl α-D-glucopyranoside	0.0	0.0	0.11
Methyl α-D-mannofuranoside	16.0	1.4	—
Methyl β-D-mannopyranoside	0.0	0.4	0.31
Methyl β-D-galactofuranoside	0.4	0.2	0.31
Methyl β-D-galactopyranoside	0.0	0.5	0.38
Methyl esters of glucose and xylose			
2-O-Methyl-D-xylose	0.0	0.0	0.39
3-O-Methyl-D-xylose	2.9	1.7	0.66
4-O-Methyl-D-xylose	0.0	0.3	0.21
5-O-Methyl-D-xylose	13.0	—	—
3,5-Di-O-methyl-D-xylose	9.3	—	—
2-O-Methyl-D-glucose	0.0	0.0	0.23
3-O-Methyl-D-glucose	1.3	1.4	0.80
4-O-Methyl-D-glucose	0.0	0.3	0.24
6-O-Methyl-D-glucose	0.5	0.96	0.80

(*Continued on p. D 3*)

TABLE 2 (*continued*)

Compound	M_M		
	E_1	E_2	E_3
Glycitols			
Glycol	0.0	0.0	—
Glyceritol	0.0	0.2	0.54
Erythritol	0.1	0.5	0.83
L-Threitol	0.3	—	0.83
erythro-Butane-2,3-diol	0.0	—	0.14
threo-Butane-2,3-diol	0.3	—	0.56
D-Ribitol	0.3	0.6	0.93
L-Arabinitol	0.6	0.9	0.96
Xylitol	0.9	0.9	0.87
D-Glucitol	1.3	1.0	0.91
D-Mannitol	1	1	1
L-Iditol	1.4	—	0.89
Galactitol	1.0	1.1	1.07
2-O-Methyl-L-arabinitol	0.1	0.1	
4-O-Methyl-L-arabinitol	0.5	0.5	
2-O-Methyl-D-xylitol	0.4	0.6	
3-O-Methyl-xylitol	0.1	0.3	
5-O-Methyl-D-xylitol	0.6	—	
1-Deoxy-D-glucitol	0.9	—	
2-O-Methyl-D-glucitol	1.2	0.8	
3-O-Methyl-D-glucitol	0.1	0.4	
4-O-Methyl-D-glucitol	1.3	0.7	
6-O-Methyl-D-glucitol	0.6	—	
6-Deoxy-L-mannitol (Rhamnitol)	0.8	—	
1,6-Di-O-acetyl-D-mannitol	0.8	—	
2-O-Methyl-D-galactitol	0.5	0.9	
3-O-Methyl-D-galactitol	1.0	0.7	
6-O-Methyl-D-galactitol	0.8	0.8	

Reduced disaccharides	*Linkage*	
Sophoritol	β (1–2)	1.2
Laminaribiitol	β (1–3)	0.1
Cellobiitol	β (1–4)	1.1
Gentiobiitol	β (1–6)	0.6

Inositols			
myo-Inositol	0.0	0.1	0.59
D-Inositol	0.0	0.4	0.70
epi-Inositol	1.8	0.9	0.81

— = Run not successful (presumed).

TABLE 3

R_F VALUES OF A NEW AMINO ACID FROM MIGNONETTE (*Reseda odorata* L), 3-(3-CARBOXY-4-HYDROXYPHENYL)-L-ALANINE

(P. O. LARSEN AND A. KJÆR, *Acta Chem. Scand.*, 16 (1962) 142)

Solvents: S_1 = Butan-1-ol–acetic acid–water (12:3:5).
S_2 = Phenol–water–conc. NH_4OH (120:30:1).
Paper: Whatman No. 1 (descending).
Temperature of run: 25°.
Detection: Ninhydrin in acetone.

Compound	R_F*		
	S_1		S_2
	a	*b*	
3-(3-Carboxy-4-hydroxyphenyl)-L-alanine (*m*-carboxy-L-tyrosine)	0.31	0.48	0.24

* Acid applied (a) in neutral solution, (b) in acid solution.

TABLE 4

R_F VALUES OF L-SERYL-L-LYSINE, O-PHOSPHORYL-L-SERYL-L-LYSINE AND O-PHOSPHORYL-L-SERYL-L-GLUTAMIC ACID

(G. FÖLSCH AND R. ÖSTERBERG, *Acta Chem. Scand.*, 15 (1961) 1963)

Solvents: S_1 = Phenol–water (80:20, v/v).
S_2 = Butan-1-ol–water (40:10:50, v/v/v).
Paper: Whatman No. 1 (S_1, ascending; S_2, descending).
Time of run: 6 h (S_1), 18 h (S_2).
Detection: D_1 = Ninhydrin (spray).
D_2 = Ammonium molybdate (spray).
(D_2 follows D_1.)

Compound	R_F	
	S_1	S_2
L-Seryl-L-lysine monohydrochloride	0.45	0.20
O-Phosphoryl-L-seryl-L-lysine dihydrochloride	0.08	0.04
O-Phosphoryl-L-seryl-L-glutamic acid	0.04	0.06

TABLE 5

R_F VALUES (THIN LAYER) OF 3-PHENYL-2-THIOHYDANTOINS OF AMINO ACIDS (PTH-AMINO ACIDS) (G. PATAKI, *Chimia (Aarau)*, 18 (1964) 24)

Solvents: S_1 = Chloroform.
S_2 = Chloroform–methanol (9:1).
S_3 = Chloroform–formic acid (100:5).
Thin layer: Kieselgel G/zinc silicate.

PTH-amino acid	R_F		
	S_1	S_2	S_3
α-Aminobutyric acid	0.26	0.79	0.54
α-Aminocaprylic acid	0.44	0.84	0.67
α-Aminoisobutyric acid	0.27	0.80	0.56
Ala	0.18	0.77	0.44
Arg	0.00	0.01	0.00
Asp	0.00	0.02	0.16
Asp-NH_2	0.00	0.34	0.09
Cit	0.00	0.34	0.08
$CySO_3H$	0.00	0.00	0.00
Glu	0.01	0.05	0.18
Glu-NH_2	0.00	0.40	0.11
Gly	0.11	0.68	0.35
His	0.01	0.40	0.01
Hypro	0.05	0.64	0.28
Ileu	0.39	0.83	0.62
Tyr	0.03	0.59	0.22
Monophenylthiourea	0.12	0.65	0.32
Diphenylthiourea	0.42	0.82	0.71
Leu	0.39	0.84	0.63
Lys	0.12	0.78	0.34
Met	0.34	0.81	0.54
Methylglutamic acid	0.23	0.82	0.50
Methionine sulphoxide	0.00	0.54	0.15
Methionine sulphone	0.02	0.59	0.17
Methylserine	0.01	0.51	0.18
Nleu	0.40	0.83	0.62
Nval	0.34	0.81	0.57
Orn	0.07	0.72	0.30
Phe	0.30	0.81	0.54
Pro	0.60	0.89	0.70
Ser	0.01	0.43	0.10
Thr	0.01	0.58	0.17
Try	0.14	0.71	0.41
Val	0.33	0.81	0.58

TABLE 6

R_F VALUES OF DINITROPYRIDYL DERIVATIVES OF AMINO ACIDS

(A. SIGNOR, L. BIONDI, M. TERBOJEVICH AND P. PAJETTA, *Gazz. Chim. Ital.*, 94 (1964) 619)

Solvents: S_1 = Toluene–pyridine–ethylene chlorohydrin–0.8 *M* ammonia (5:1:3:3).
S_2 = Butan-1-ol–3% ammonia (1:1).
S_3 = Isoamyl alcohol–phenol–water (1:1:1).
S_4 = 1.5 *M* phosphate buffer, pH 6.
Paper: Whatman No. 1.
Temperature: 21°.

Compound	R_F			
	S_1	S_2	S_3	S_4
α-N-DNPyr-arginine	0.33	0.30	0.82	0.52
α-N-DNPyr-histidine	0.13	0.21	0.54	0.57
DNPyr-aspartic acid	0.00	0.02	0.02	0.66
DNPyr-cysteic acid	0.00	0.00	0.00	0.68
DNPyr-glutamic acid	0.00	0.02	0.02	0.62
DNPyr-alanine	0.25	0.32	0.52	0.48
DNPyr-asparagine	0.07	0.10	0.23	0.48
DNPyr-glycine	0.17	0.19	0.34	0.43
DNPyr-phenylalanine	0.64	0.51	0.84	0.32
DNPyr-glutamine	0.07	0.13	0.29	0.48
DNPyr-isoleucine	0.68	0.56	0.76	0.44
DNPyr-lysine	0.76	0.56	0.82	0.06
DNPyr-leucine	0.68	0.56	0.81	0.45
DNPyr-proline	0.20	0.30	0.59	0.55
DNPyr-serine	0.12	0.17	0.30	0.53
DNPyr-threonine	0.15	0.23	0.35	0.57
DNPyr-tryptophan	0.56	0.46	0.74	0.14
DNPyr-valine	0.47	0.45	0.73	0.48
DNPyr-amine	0.93	0.78	0.92	0.08
DNPyr-phenol	0.24	0.35	0.20	0.40

TABLE 7

CORRECTED ELECTROPHORETIC MOBILITIES OF SOME METHYLATED CYCLITOLS IN SODIUM ARSENITE

(J. L. FRAHN, *Australian J. Chem.*, 17 (1964) 274)

Buffer: 0.2 *M* sodium arsenite at pH 9.6.
The following values should be used instead of the earlier ones (J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65).

Compound	M_R
Pinitol	0.25
Quebrachitol	0.15
Bornesitol	0.11
5-O-Methyl-myoinositol	0.15

TABLE 8

THIN-LAYER CHROMATOGRAPHY OF THUJONES AND THUJYL ALCOHOLS

(G. M. NANO AND A. MARTELLI, *Gazz. Chim. Ital.*, 94 (1964) 816)

Solvents: S_1 = Benzene (single development).
S_2 = Benzene (double development).
S_3 = Benzene (triple development).
S_4 = 1.5% Methanol in benzene.
Thin layer: Kieselgel G (Merck).

Compound	R_T*			
	S_1	S_2	S_3	S_4
iso-Thujyl alcohol	0.28	0.31	0.45	0.275
Thujyl alcohol	0.31	0.35	0.49	0.31
neo-Thujyl alcohol	0.43	0.475	0.63	0.42
neoiso-Thujyl alcohol	0.58	0.645	0.77	0.56
α-Thujone	0.95	0.98	0.96	0.98
β-Thujone	1.00	1.00	1.00	1.00

* R_T = Mobility relative to β-thujone.

TABLE 9

R_F VALUES OF SOME SUBSTANCES RELATED TO TANNINS

(K. K. REDDY, S. RAJADURAI, K. N. S. SASTRY AND Y. NAYUDAMMA, *Australian J. Chem.*, 17 (1964) 238)

Solvents: S_1 = 6% Acetic acid.
S_2 = Butan-2-ol–acetic acid–water (14:1:5, v/v).
S_3 = 6% Acetic acid, containing 2.0% (w/v) formic acid.
S_4 = Benzyl alcohol–isopropanol–*tert.*-butanol–water (3:1:1, v/v), containing 1.8% (w/v) formic acid.
Paper: Whatman No. 1.

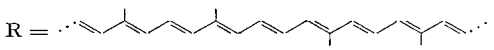
Compound	R_F			
	S_1	S_2	S_3	S_4
Corilagin	0.45	0.29	0.44	0.25
Gallotannin	0.21	0.58	0.23	0.41
Glucose	—	0.24	—	—
β-Penta-O-galloylglucose	0.22	0.58	0.21	0.41
Tetra-O-galloyl-β-glucose	0.33	0.62	0.34	0.44
Gallic acid	0.46	0.72	0.49	0.57
Methyl gallate	0.55	0.88	0.54	0.70
Gallic acid	0.46	0.72	0.49	0.57

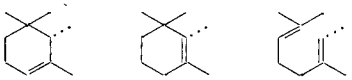
TABLE 10

R_F VALUES (THIN LAYER) OF PURE ALL-*trans*-CAROTENES

(H. R. BOLLIGER, A. KÖNIG AND U. SCHWIETER, *Chimia (Aarau)*, 18 (1964) 136)

Solvents: S_1 = Light petroleum (90–110°)–benzene (9:1).
S_2 = Light petroleum (90–110°)–benzene (5:5).
S_3 = Light petroleum (90–110°)–benzene (1:9).
Thin layer: "Darlington" light magnesium oxide, activated for 1 h at 120°.

R = 



α β γ

Compound	*Structure*	R_F		
		S_1	S_2	S_3
ε-Carotene	α + R + α	0.47	0.70	0.84
α-Carotene	α + R + β	0.26	0.66	0.80
β-Carotene	β + R + β	0.11	0.49	0.74
δ-Carotene	α + R + γ	0.00	0.20	0.55
γ-Carotene	β + R + γ	0.00	0.11	0.41
Lycopene	γ + R + γ	0.00	0.00–0.02	0.13

TABLE 11

R_F VALUES OF SOME BACTERIAL CAROTENOIDS

(L. M. JACKMAN AND S. L. JENSEN, *Acta Chem. Scand.*, 15 (1961) 2058)

Solvent: 10% Acetone in petroleum ether.
Paper: Schleicher & Schüll No. 287 (A. JENSEN AND S. L. JENSEN, *Acta Chem. Scand.*, 13 (1959) 1863).
Detection: Not specified.

Compound	R_F
trans-3,4-Dehydro-rhodopin	0.70
trans-Rhodovibrin	0.54

TABLE 12

R_F VALUES OF INTERMEDIATE PRODUCTS IN THE SYNTHESIS OF EPHEDRINE

(K. MACEK, S. VANĚČEK AND H. BEČVÁŘOVÁ, *Collection Czech. Chem. Commun.*, 29 (1964) 313)

Solvents: S_1 = Formamide/benzene–cyclohexane (1:9).
S_2 = Formamide/benzene–cyclohexane (3:7).

Compound	R_F		
	Free compound	*2,4-Dinitrophenylhydrazone*	*3,5-Dinitrobenzoate*
	S_1	S_2	S_1
Phenyl-acetyl-carbinol	0.20	0.38	0.50
Methyl-benzoyl-carbinol	0.30	0.25	0.50
Methyl phenyl diketone	0.83	0.90	—
Benzaldehyde	—	0.87	—
Acetoin	—	0.13	0.09
Diacetyl	—	0.53	—
Benzyl alcohol	—	—	0.77

TABLE 13

RELATIVE ELUTION VOLUMES (GAS CHROMATOGRAPHY) OF INTERMEDIATE PRODUCTS IN THE SYNTHESIS OF EPHEDRINE

(K. MACEK, S. VANĚČEK AND H. BEČVÁŘOVÁ, *Collection Czech. Chem. Commun.*, 29 (1964) 313)

Apparatus: Griffin VPC 2B.
Column: 180 cm glass column, packed with Celite 545 (60–80 mesh).
Stationary phase: 30% Silicone Elastomer E 301.
20% Reoplex 400.
Helium flow-rate: 33 ml/min.
Elution volumes relative to benzyl alcohol = 1.00.

Compound	*R*	
	Silicone 170°C	*Reoplex 190°C*
Methyl-benzoyl-carbinol	2.44	2.59
Phenyl-acetyl-carbinol	2.50	2.58
Methyl phenyl diketone	1.78	1.00
Benzyl alcohol	1.00	1.00
Benzaldehyde	0.74	0.39
Acetoin	0.21	0.16

TABLE 14

RELATIVE RETENTION TIMES (GAS CHROMATOGRAPHY) OF SOME TOCOPHEROLS

(M. KOFLER, P. F. SOMMER, H. R. BOLLIGER, B. SCHMIDLI AND M. VECCHI, in R. S. HARRIS AND I. G. WOOL (Editors), *Vitamins and Hormones*, Vol. 20, Academic Press, New York, 1962, p. 432)

Apparatus: Pye Argon Chromatograph.
Column: 2 m glass column, packed with Celite (100–120 mesh).
Stationary phase: 5% Apiezon N high vacuum grease.
Temperature: 260°.
Argon flow-rate: 60 ml/min.
Retention times relative to n-$C_{28}H_{58}$ = 1.00.

Compound	*R*
α-Tocopherol	2.34
ζ_2-Tocopherol	1.88
γ-Tocopherol	1.76
β-Tocopherol	1.68
5-Methyltocol	1.50
η-Tocopherol	1.41
δ-Tocopherol	1.25
Tocol	1.10
n-$C_{28}H_{58}$	1.00

TABLE 15

R_F VALUES (RELATIVE) OF SISAUSTRICIN AND ISOMERIDES

(A. KJÆR AND B. W. CHRISTENSEN, *Acta Chem. Scand.*, 16 (1962) 71)

Solvents: S_1 = Benzene–heptane–water (9:2:9).
S_2 = Carbon tetrachloride–30% acetic acid (1:1) (Ž. PROCHÁZKA, V. ŠANDA AND L. JIROUSEK, *Collection Czech. Chem. Commun.*, 27 (1962) 94).
Paper: Not specified.
Detection: Grote's reagent (spray).

Compound	R_{Ph}*	
	S_1	S_2
4,5-Dimethyl-2-oxazolidinethione	0.56	0.41
(±)-5-Ethyl-2-oxazolidinethione	0.62	0.47
(±)-4-Ethyl-2-oxazolidinethione	0.66	0.55
Sisaustricin [(+)-4-ethyl-2-oxazolidinethione]	0.66	0.55

* R_{Ph} = R_F compound/R_F (±)-5-phenyl-2-oxazolidinethione (A. KJÆR AND R. GMELIN, *Acta Chem. Scand.*, 11 (1957) 906).

TABLE 16

R_F VALUES (THIN LAYER) OF ERGOT ALKALOIDS

(J. L. McLAUGHLIN, J. E. GOYAN AND G. PAUL, *J. Pharm. Sci.*, 53 (1964) 308)

Solvents: S_1 = Ethyl acetate–N,N-dimethylformamide–ethanol (130:19:1) on Silica Gel G.
S_2 = Benzene–N,N-dimethylformamide (13:2) on Silica Gel G.
S_3 = Chloroform–ethyl ether–water (175:25:50) on Aluminium Oxide G.
S_4 = Chloroform–ethyl ether–water (3:1:1) on Aluminium Oxide G.

Alkaloid	R_F			
	S_1	S_2	S_3	S_4
Ergonovine	0.17	0.12	0.00	—
Ergometrinine	0.44	0.38	0.01	—
Ergotamine	0.31	0.31	0.01	—
Ergotaminine	0.68	0.64	0.07	—
Ergosine	0.35	0.31	0.02	—
Ergosinine	0.75	0.68	0.12	—
Ergocristine	0.54	0.56	0.09	—
Ergocristinine	0.80	0.74	0.38	—
Ergocornine	0.58	0.59	0.10	0.24
Ergocorninine	0.83	0.73	0.31	—
Ergocryptine	0.60	0.55	0.15	0.30
Ergocryptinine	0.85	0.75	0.46	—

TABLE 17

R_F VALUES (THIN LAYER) OF HYDROGENATED ERGOT ALKALOIDS

(T. HOHMANN AND H. ROCHELMEYER, *Arch. Pharm.*, 297 (1964) 187)

Solvent: Formamide/ethyl acetate–*n*-heptane–diethylamine (250:300:1).
Thin layer: Cellulose layers were impregnated with 15% formamide in acetone.

*Alkaloid**	R_F
Dihydroergotamine	0.09
(Ergotamine	0.11)
(Ergosine	0.17)
Dihydroergocristine	0.30
Dihydroergocornine	0.38
Dihydroergocryptine	0.50
(Ergocristine	0.41)
(Ergocornine	0.50)
(Ergocryptine	0.61)

* The alkaloids in parentheses are not hydrogenated ergot alkaloids.

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