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ADSORPTION STUDIES ON PORAPAK S AT VARIOUS TEMPERATURES BY GAS CHROMATOGRAPHIC AND STATIC METHODS

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SUMMARY

The distribution coefficients of Ar, CH_4, CO_2, O_2 and N_2 in Porapak S were obtained by Gas chromatography and by adsorption studies at various temperatures and a comparison was made between the two methods. Discontinuities in the curves are explained by a glassy transition in the polymer support.

INTRODUCTION

In the previous two articles dealing with the study that has been carried out on a 200 ft., $\frac{1}{8}$ in. O.D., Porapak S (50–80 mesh) column[1,2] we have discussed (1) the effect of the carrier gas' nonideality and adsorption on the net retention volume; (2) the gas flow properties of this type of column. This final article is concerned with the temperature dependence of (a) the net retention volume (obtained by the gas chromatographic (GC) method) and (b) the distribution coefficient (obtained by the static method). For convenience of discussion, this article is separated into two parts one dealing with the GC system and the other with the static system.

GAS CHROMATOGRAPHIC SYSTEM

Theoretical

It is a simple matter to show that the partition or distribution coefficient (K) is related to the partial molar free energy (ΔG^0) by

$$\Delta G^0 = -RT \ln K \tag{1}$$

For a gas–solid GC system, K is related to the net retention volume V_N by

$$V_N = KW_s \tag{2}$$

where W_s is the weight of the packing material. As it has been pointed out in the previous article[1], eqn. 2 can also be expressed in terms of surface area (S) accompanied

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by the appropriate change in the units of K. Substitution of eqn. 2 into eqn. 1 gives

$$\Delta G^0 = -RT \ln V_N + RT \ln W_s \tag{3}$$

But since

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{4}$$

then after this substitution and rearrangement we can write

$$\ln V_N = \frac{-\Delta H^0}{RT} + q \tag{5}$$

where

$$q = \frac{\Delta S^0}{R} + \ln W_s \tag{6}$$

As according to eqn. 5, a plot of $\ln V_N$ or under suitable conditions $\ln t_R$ against $1/T$ (where t_R is the retention time) should give a straight line from whose slope ΔH^0 can be extracted[3-8]. Furthermore, if K is known then both ΔS^0 and ΔG^0 can also be calculated.

Experimental apparatus and procedure

The experimental apparatus and procedure has already been described in the first article[1], but the present experiments were carried out under the following set of conditions:

Carrier gas, helium; temperature bath, (a) $t = 0°$ (ice–water mixture) and (b) $t > 0°$ (paraffin oil and heater); detector, electron capture (used in the "helium detector" mode).

Results and discussion

A sample (*ca.* 0.45 μl) containing approximately equal parts of H_2, N_2, O_2, Ar, CH_4 and CO_2 was subjected to a gas chromatographic separation at various column temperatures. The net retention volumes were calculated by the method described in the previous article and are given in Table I. The log V_N *vs.* $1/T$ plots for N_2, O_2, and Ar are shown in Fig. 1 and for CH_4 and CO_2 in Fig. 2 and Fig. 3, respectively. A plot similar (though not included) was obtained for H_2.

TABLE I

NET RETENTION VOLUMES OF VARIOUS GASES AT DIFFERENT TEMPERATURES

$V_N(CO_2)/V_N(CH_4) = 5.99$ at $T = 273°K$.

Gas	$T = 351°K$[a]	$T = 335°K$[a]	$T = 320°K$[a]	$T = 306°K$[a]	$T = 273°K$[a]
H_2	8.4	9.0	9.6	10.3	12.4
N_2	19.2	22.1	25.6	29.6	46.2
Ar	24.8	28.9	33.5	39.9	62.3
O_2	26.0	30.0	34.4	38.9	56.6
CH_4	53.3	65.5	81.4	103.1	210.1
CO_2	139.9	193.0	273.8	400.1	1258.2

[a] $\pm \frac{1}{2}°$.

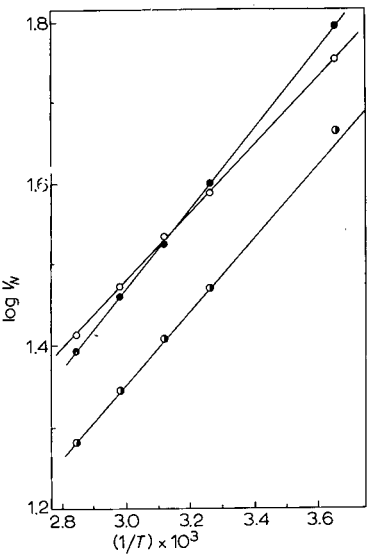

Fig. 1. Plot of log V_N against $1/T$ for N_2, Ar, and O_2. ◑ = N_2; ○ = O_2; ● = Ar.

On the whole, the results expressed in Figs. 1, 2 and 3 are more or less typical. There are however two points which should be noted. The first is found in Fig. 1. The crossing of the Ar and O_2 plots indicates that at $1/T = 3.2 \times 10^{-3}$ ($\sim 316°K$) both Ar and O_2 have the same nett retention volume and that the separation of these two constituents at this temperature is impossible on this column. Also, by working either above or below this temperature the elution order can be reversed.

The second and perhaps the more important point refers to the N_2, CH_4, and

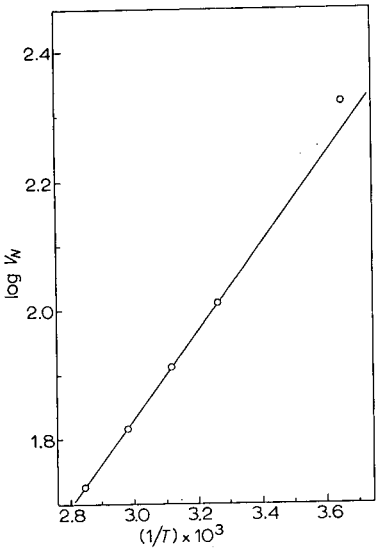

Fig. 2. Plot of log V_N against $1/T$ for CH_4.

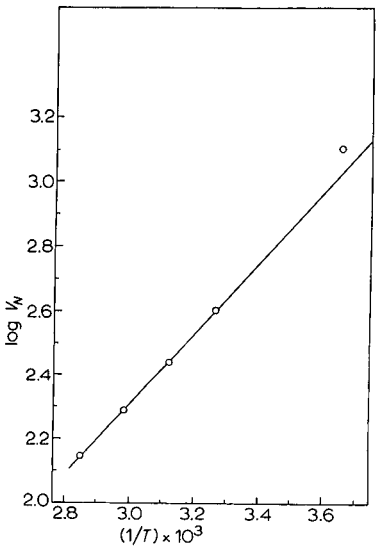

Fig. 3. Plot of log V_N against $1/T$ for CO_2.

CO_2 plots. It can be noted that there exists a rather large deviation at $1/T = 3.66 \times 10^{-3}$ (273°K). Repeated experiments (CH_4 and CO_2 case) showed very high degree of reproducibility at this temperature (the spread in the experimental data would be insignificant in this type of a plot) and consequently these deviations cannot be attributed to experimental error, and will be discussed later when other facts are presented.

The ΔH^0 and q values were calculated by the least squares method from the linear portions of the plots and are tabulated in Table II.

K(CH_4) AND K(CO_2) AS A FUNCTION OF T BY THE STATIC METHOD

Introduction

The large deviations found at 273°K made it imperative that the temperature range should be extended below 273°. This however was not to be a simple task since even at 273°K using moderate carrier gas flow rates, the retention times of CH_4 and CO_2 were about 1 and 6.5 h, respectively. A further drop of 20° would at least double

TABLE II

THE ΔH^0 AND THE q VALUES FOR VARIOUS GAS SAMPLES USING HELIUM AS THE CARRIER GAS

Gas	ΔH^0 *(calc.)*	q
H_2	−940	0.7858
N_2	−2063	−0.0026
Ar	−2239	−0.0009
O_2	−1917	0.5156
CH_4	−3125	−0.5087
CO_2	−4984	−2.2148

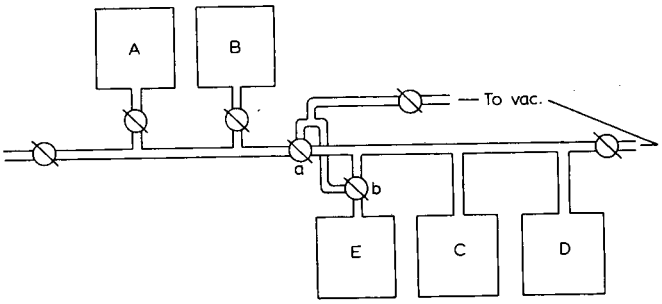

Fig. 4. Block diagram of the static system.

or perhaps even triple these retention times, making the study impractical. A static method could be made to serve a dual purpose. It would allow the study to be extended to the lower temperature region, and could serve as an independent check on the thermodynamic date obtained by the GC method.

Experimental

The apparatus used in this study consisted of a typical high vacuum glass

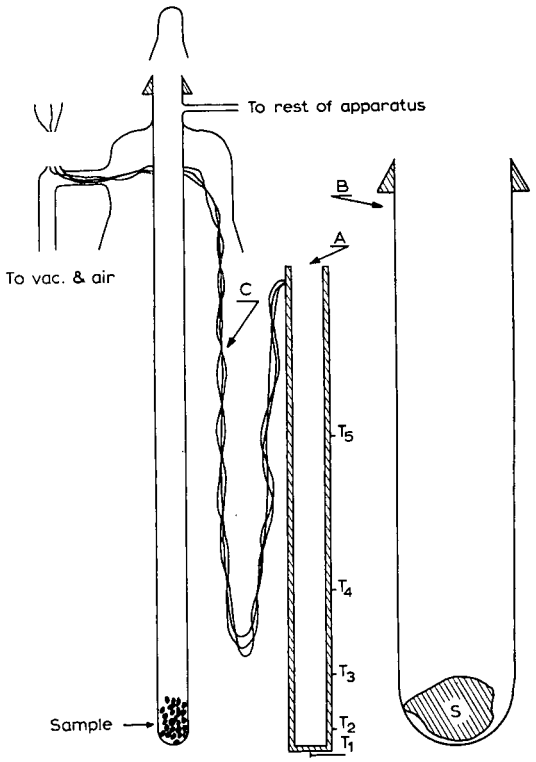

Fig. 5. Modified LEROY still. A = brass tube on which the sample heating coil and thermocouples are mounted; B = outer jacket; C = heating and thermocouple wires; T_1, T_2, T_3, T_4, T_5 = thermocouples; S = styrofoam.

apparatus. For clarity, only the essential parts are shown in a block form in Fig. 4. A and B are each 1-l gas storage bulbs. C is a modified LEROY still[9] which could be assembled or disassembled so that solid material could be introduced into it, degassed and studied within it. A more detailed description of the LEROY still is shown in Fig. 5. The outside jacket and the brass tube (closed at the bottom end) on which the heating coil and the thermocouples were mounted could be removed prior to degassing. The role of the styrofoam was to ensure a good contact between the sample tube and the brass jacket when the still was assembled.

D is a thermocouple pressure gauge (R.C.A.-model 1946). During operation it was maintained at 0°C with ice–water mixture. A 6-V battery served as a source of constant current and a Heathkit millivolt source (Model EUW-16) was used to buck the e.m.f. so that a 12 MV signal (stick vacuum) could be accommodated on the 5 MV span of a potentiometric recorder. The purpose of this gauge was only to indicate whether or not equilibrium conditions had been reached.

E is a McLeod gauge with the lowest limit of pressure indications of *ca.* 10^{-5} T. The volume of the McLeod gauge (mercury in the down position) was calibrated so that it may serve both as a pressure measuring device and as a constant known volume. The presence of mercury necessitated this calibration to be carried out at low pressure, *ca.* 0.1 mm pressure of helium gas.

The volume including the sample tube (the inner tube of the LEROY still), the thermocouple pressure gauge, and the interconnecting tubing was also calibrated. Stopcocks a and b were both three way stopcocks, as indicated in the diagram.

Both CO_2 and CH_4 were degassed by a number of repeated freeze-pump-expand cycles before they were stored in the storage bulbs.

The solid sample was 0.442 g of the identical material which was used in the column preparation. The treatment of this material prior to use has been described[1].

Experimental procedure

Prior to each experiment, the solid sample was heated under vacuum at 180°C for approximately 12 h. After the entire system (except storage bulbs A and B) was evacuated, the gas which was contained in one of the storage bulbs was allowed to expand only into the McLeod gauge (E) by means of stopcocks a and b. After sufficient time was allowed for pressure equilibration (about 15–20 min) the pressure was then measured with the McLeod gauge. In the event the pressure was higher than required, some of the gas was vented out of the system. The McLeod gauge was then brought to its down position and after 15–20 min stopcocks a and b and the one leading to the storage bulb were turned off. It should be pointed out that the 15–20 min pressure equilibration time was required in order to get good reproducibility in the pressure measurements.

The LEROY still and the sample contained within were cooled down to about −190°C after which the air jacket was evacuated. The gas contained in the McLeod gauge was then allowed to expand only into chambers C and D and the interconnecting tubing by means of stopcock b. The pressure was monitored with the thermocouple pressure gauge. After the gas had frozen out on the solid sample, the final pressure was checked with the McLeod gauge.

The temperature of the sample was raised by applying a predetermined voltage to the heating coils of the LEROY still. The temperature and pressure were monitored

on the recorder, and when equilibrium conditions were reached, at least six pressure measurements were made with the McLeod gauge. The temperature was then raised further and the cycle repeated. It should be mentioned that it required about 1.5 h to reach each temperature and pressure equilibrium.

Calculations of K

If one operates in a very low pressure region, it can be assumed that (a) Henry's Law is obeyed; (b) the gas behaves ideally; (c) the distribution coefficient (K) can be expressed in terms of concentrations[10], that is

$$K = C_s/C_m = \left(\frac{N_s}{W_s}\right)\Big/\left(\frac{N_x}{V_x}\right) \tag{7}$$

where

W_s is the weight of the adsorbent

N_s is the number of moles of gas adsorbed on the surface at some temperature T_x

V_x is the volume containing the non adsorbed gas which is also at temperature T_x

N_x is the number of moles of gas contained in V_x.

With the aid of the ideal gas law, eqn. 7 can be rewritten to read

$$K = \frac{N_s}{W_s} \cdot \frac{RT_x}{P} \tag{8}$$

Since R and W_s are known constants, and T_x and P are measurable variables, it remains now to determine N_s. This can be done if one knows the initial number of moles of gas that are introduced into the system and the final number of moles of non-adsorbed gas after equilibration since

$$N_s = N_0 - N_f \tag{9}$$

where o and f refer to the initial and the final quantities. N_0 like W_s is a known constant adjusted by the experimenter. N_f on the other hand is not directly accessible and can only be calculated from the two variables T_x and P. The problem now reduces to relating N_f to P and T_x.

If the entire system was to be kept at the same temperature T_x, then it would be a simple matter to relate N_f to P and T_x. Generally this is not the case and consequently there will be at least one temperature gradient present in the system. The present system has two temperature gradients (one between the LEROY still and the interconnecting tubing and the other between the thermocouple pressure gauge and the interconnecting tubing), but other systems may have more. The presence of these gradients and the different temperature that may be found in the system, make it necessary to discuss the system in parts.

At any particular point in the system where the temperature is T_i, the number of moles of gas bounded by the infinitesimal volume dV is

$$dN = \frac{P}{R} \cdot \frac{dV}{T_i} \tag{10}$$

If the temperature T_i is constant over a volume V_i, then integration of eqn. 10 gives

$$N_i = \frac{P}{R} \cdot \frac{V_i}{T_i} = a_i \frac{P}{R} \tag{11}$$

Also, if T_i remains constant for the duration of the entire experiment, then a_i can be considered as a constant of the system. This is true for all parts of the system where the above conditions are met. In the case where $T_i = T_x$; a_x is a variable and it is preferable to express N_x by

$$\frac{P}{R} \cdot \frac{V_x}{T_x}$$

The number of moles contained in the volume defined by the temperature gradient is

$$N_i = \frac{P}{R} \int \frac{dV}{T} \tag{12}$$

Now, if the extreme temperatures of the gradient are held constant, so will be the volume. No matter how complex the integral may be, the final expression will be

$$N_i = b_i \frac{P}{R} \tag{13}$$

where all b_i like a_i will be constants of the system. Finally, the number of moles of gas contained in the volume V defined by the temperature gradient between T_x and say T (the ambient temperature), will be some function of T_x. The exact function may vary from system to system but if V is kept constant, we can write that

$$n = \frac{PV}{R} f(T_a, T_x) \tag{14}$$

where $f(T_a, T_x)$ is some function involving both T_a and T_x.

N_f can now be expressed in terms of the various contributions and in turn eqn. 9 can be rewritten to give

$$N_s = N_0 - \frac{P}{R} \left[\Sigma a_i + \Sigma b_i + \frac{V_x}{T_x} + V f(T_a, T_x)\right] \tag{15}$$

In practice it would require considerable effort to determine V and $f(T_a, T_x)$. It is preferable then to minimize the last term of eqn. 15 to the point where it can be neglected. This can be done by designing the apparatus so that V constitutes about 1% or less of the entire volume. If these conditions are met, eqn. 15 reduces to

$$N_s = N_0 - \frac{P}{R} F_x \tag{16}$$

where

$$F_x = k + \frac{V_x}{T_x} \tag{17}$$

and

$$k = \Sigma a_i + \Sigma b_i \tag{18}$$

V_x and k can be determined from a non-sorbing gas such as helium. Since the gas is non-sorbing, $N_s = 0$ and eqn. 16 can be rewritten to give

$$\frac{N_0 R}{P} = F_x \tag{19}$$

If the assumptions made leading to eqn. 16 are correct, a plot of N_0R/P *vs.* $1/T_x$ should give a straight line with an intercept equal to k and with a slope of V_x.

It remains now to substitute eqn. 16 into eqn. 8 in order to get the final expression for K, that is

$$K = \frac{1}{W_s}\left[\frac{N_0 R}{P} - F_x\right] T_x \tag{20}$$

RESULTS AND DISCUSSION

In the present system the volume V was small (2–3 ml) compared to the entire volume (about 500 ml). Calculations showed that if a linear temperature gradient was assumed along a tube of uniform cross-sectional area, then

$$\mathrm{f}(T_a, T_x) = \frac{1}{(T_a - T_x)} \ln \frac{T_a}{T_x} \tag{21}$$

and that on the average the $V\mathrm{f}(T_a, T_x)$ term would contribute only about 0.5% with respect to the other two terms namely k and V_x/T_x. Under these conditions the omission of the $V\mathrm{f}(T_a, T_x)$ term is justified.

In the first article[1], it was shown that at elevated pressures and a 273°K there is a measurable interaction between helium and the solid material under investigation. In order to minimize this interaction, the system was calibrated (V_x and k determined) with helium under low pressure. On the average the helium pressure was about 3×10^{-2} T. The data was plotted as $(N_0R/P)T_x$ *vs.* T_x and is shown in Fig. 6. Under these conditions k is the slope and V_x is the intercept. As can be seen the plot is linear indicating that the assumptions made are reasonable.

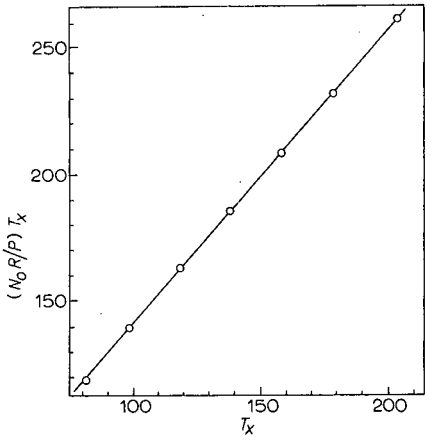

Fig. 6. $(N_0R/P)T_x$ as a function of T_x.

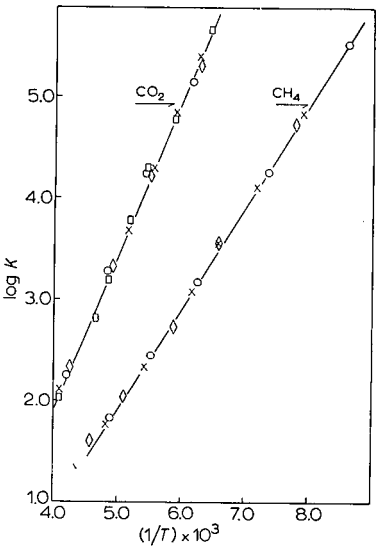

Fig. 7. Plot of log K against $1/T$ for CH_4 and CO_2.
CH_4: , ◇, $P_0 = 23.03 \times 10^{-3}$ mm; ○, $P_0 = 51.49 \times 10^{-3}$ mm; ×, $P_0 = 88.62 \times 10^{-3}$ mm.
CO_2: □, $P_0 = 23.40 \times 10^{-3}$ mm; ○, $P_0 = 54.92 \times 10^{-3}$ mm; ×, $P_0 = 97.10 \times 10^{-3}$ mm; ◇, $P_0 = 136.10 \times 10^{-3}$ mm.

The distribution coefficients of CH_4 and CO_2 were measured in the temperature range 116° to 240°K with a fourfold initial pressure (P_0) variation and 155° to 240°K with a sixfold P_0 variation, respectively. The initial pressure refers to the pressure of the gas in the McLeod gauge before it is allowed to expand into the rest of the system. The lower temperature limit is governed by the appearance of sufficient gas pressure so that accurate pressure measurements can be made.

The results of these experiments are summarized in Fig. 7 where log K is plotted against $1/T$. Both lines are linear and in each case the variation of P_0 does not seem to affect the distribution coefficients indicating that under these low pressure conditions the adsorption isotherms are essentially linear. From the least square, the two lines can be characterized as follows:

	CH_4	CO_2
slope	975.7	1,502
intercept	−2.942	−4.095

Extrapolation to 273°K gave the following distribution coefficients $K(CH_4) = 4.25$ ml/g; $K(CO_2) = 25.44$ ml/g.
The ratio of $K(CO_2)/K(CH_4)$ is then 5.99. From the GC study it was found that at 273°K, $V_N(CO_2)/V_N(CH_4)$ was also 5.99. Although these ratios are in excellent agreement, it is more important to determine how the GC and the static system data correspond numerically. This can be tested utilizing eqn. 2 which relates K and V_N. Calculations show that the total weight of the packing material in the column (W_s) should be 49.3 g. Unfortunately due to several accidents during the column prepa-

ration the exact weight of the material is not known, but from the bulk density measurements and the volume of the empty column (calculated assuming uniform cross sectional area) this quantity was calculated to be 48.6 g. The close agreement between these two values adds credance to the following: (a) the method of determining V_m as described in the first article[1]; (b) the assumptions made leading to eqn. 20; (c) the method of determining V_x and k; (d) the deviations observed at 273° in Figs. 1, 2 and 3.

The ΔH^0 values were calculated from the slopes of the two lines and are compared below with those obtained from the GC data.

	Static	GC
$\Delta H^0(CH_4)$(kcal)	4.46	3.12
$\Delta H^0(CO_2)$(kcal)	6.87	5.00

On first inspection it appears that the static system gives higher ΔH^0, but since (as it has been shown) the two systems give virtually identical results this difference cannot be a by-product of the systems but must result from the difference in the temperature range employed. What this implies is that there is a change in the partial molar heat of adsorption as one goes from one temperature range to the other. This point is well supported by the GC data obtained at 273°K, in that the data does not fall on the ln V_N *vs.* $1/T$ line, but when it is converted to log K, it fits well the extrapolated log K *vs.* $1/T$ line obtained from the static system. This then means that the change of slope occurs somewhere just above 273°K. This transition point was determined by first converting ln V_N to log K or *vice versa* and calculating where the two lines intersect. The CH_4 and the CO_2 data show these temperatures to be 284.7° and 286.6°K, respectively. Again the agreement is very good with the average value at 285.6 ± 1°K. However, this transition could not be detected on a differential scanning calorimeter (Perkin-Elmer Model DSC-1B). The reason for this may be that the transition is slow and/or the heat of transition is negligible. The temperature at which this transition occurs and the inability to observe this transition on the calorimeter strongly suggest this to be the β-transition which for polystyrene ranges from 5° to 25°C (ref. 11).

In Table III are the ΔG^0 and the ΔS^0 values as computed at 273°K. The ΔS^0

TABLE III

THE ΔG^0 AND THE ΔS^0 VALUES FOR VARIOUS GASES AT 273°K

Gas	*ΔG^0(Kcal.)*	*ΔS^0(e.u.)*
Gas chromatography	(Helium carrier gas)	
H_2	0.762	−6.23
N_2	0.048	−7.80
Ar	−0.114	−7.80
O_2	−0.062	−6.77
CH_4	−0.790	−13.44
CO_2	−1.746	−18.71
Static method		
CH_4	−0.790	−13.46
CO_2	−1.751	−18.73

values for CH_4 and CO_2 (GC) were calculated using the ΔH^0 values obtained from the static system since the experimental points fall on the steeper part of the ln V_N or log K *vs.* $1/T$ plot. There is some error in the ΔS^0 for N_2 since the ΔH^0 obtained from the higher temperature region was used in the calculation.

It thus appears possible to use gas chromatography to determine the temperature of glassy state transitions in polymers.

REFERENCES

1 J. J. CZUBRYT, H. D. GESSER AND E. BOCK, *J. Chromatogr.*, 53 (1970) 439.
2 J. J. CZUBRYT AND H. D. GESSER, *J. Chromatogr.*, 53 (1970) 453.
3 S. A. GREEN AND H. PUST, *J. Phys. Chem.*, 62 (1958) 55.
4 R. S. PETROVA, E .V. KHRAPOVA AND K. D. SHCHERBAKOVA, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 18.
5 C. G. SCOTT, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 36.
6 R. L. GALE AND R. A. BEEBE, *J. Phys. Chem.*, 68 (1964) 555.
7 J. KING, JR. AND S. W. BENSON, *Anal. Chem.*, 38 (1966) 261.
8 G. L. HARGROVE AND D. T. SAWYER, *Anal. Chem.*, 40 (1968) 409.
9 D. J. LEROY, *Can. J. Res.*, B29 (1950) 492.
10 D. C. LOCKE, *J. Phys. Chem.*, 69 (1965) 3768.
11 R. F. BOYER, *160th ACS Meeting, September, 1970, Organic Coatings and Plastics, Chemistry Papers*, 30, No. 2 (1970) p. 1.

CHROM. 5369

THE USE OF VANADIUM(II), MANGANESE(II), AND COBALT(II) CHLORIDES AS PACKINGS TO SEPARATE ALKANES, ALKENES, AND ALKYNES BY GAS CHROMATOGRAPHY

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SUMMARY

The anhydrous chlorides of vanadium(II), manganese(II) and cobalt(II) were studied for possible use as packings in gas–solid chromatography. These compounds differ primarily in their number of available "3d" electrons, *viz.* V(II)($3d^3$), Mn(II)-($3d^5$) and Co(II)($3d^7$). Various saturated and unsaturated organic compounds were investigated as adsorbates to observe the influence of varying π-electron densities. The heats of sorption were calculated and found to vary directly with the π-electron density of the adsorbate and vary inversely with the number of 3d electrons of the adsorbent. Separations achieved were a result of the π-electron density of the samples. Conjugated systems adsorbed on the salts were studied, but the column appeared to be best utilized for compounds having isolated π-bonds. A highly electronegative group, not near to a π-bond, appeared to have little effect on the degree of sorption. Although it was not our purpose to determine absolutely the mechanism of sorption, our data indicate that chemisorption plays a major role in the interaction between adsorbent and adsorbate.

INTRODUCTION

GARNER AND VEAL[1] and later GARNER[2] defined three processes, each of which could be termed adsorption. The first of these, physical adsorption, is due primarily to Van der Waals forces. The latter two processes, reversible chemisorption and irreversible chemisorption, are attributed to bond formation between the adsorbent and the adsorbate. KIPLING AND PEAKALL[3] observed that no single experimental measurement could provide a criterion to distinguish between chemisorption and physical adsorption as there were many exceptions to the general concept that the heat of sorption for physical adsorption is less than that for chemisorption. In a chemisorption process all three operations generally occur (unless conditions are

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rigidly controlled) and the determined heats of sorption reflect the average contribution of all three sorption processes.

Many publications have appeared wherein the authors proposed chemisorption as the mechanism involving the sorption of unsaturated compounds onto transition metal salts[4–11]. DENT AND KOHES[12] proposed a definite bond between propylene and zinc oxide, infrared spectroscopy being used to study the sorption process. Some applications to gas chromatography have been demonstrated[13–15] using various transition metal compounds as adsorbents. In a series of articles, GIL-AV *et al.*[16–19] demonstrated the use of stationary phases containing silver nitrate to perform separations of saturated and unsaturated compounds.

These studies, coupled with the potential of column selectivity and high-temperature operation, formed the basis of our research. It was hoped that the chlorides of vanadium(II), manganese(II) and cobalt(II) would be effective as packings in the separation of various unsaturated compounds by gas–solid chromatography (GSC).

EXPERIMENTAL

Column preparation

V(II), Mn(II) and Co(II) chlorides were purchased, as Reagent Grade anhydrous salts, from K and K Laboratories, Plainview, N.Y. DSC studies indicated the presence of hydrated water and curing conditions were selected to assure the anhydrous state. The columns were glass, 6 ft. in length, 0.25 in. O.D. Each was packed with 60/80 mesh salt using vacuum and slight vibration. The packings were cured in the columns under helium flow for three days at the following temperatures: VCl_2 (160°), $MnCl_2$ (230°) and $CoCl_2$ (190°). After three days, the columns were again vibrated under vacuum to fill any voids and cured an additional day.

Apparatus

A Varian Aerograph Model 1840-1 gas chromatograph equipped with dual columns, flame ionization detectors, and on-column injection ports was employed with helium, dried over calcium chloride and molecular sieves, as the carrier gas.

A Leeds and Northrup potentiometer equipped with an iron–constantan thermocouple served as low-temperature monitoring system.

10-μl-capacity Hamilton syringes, obtained from Hamilton Co., Whittier, Calif., U.S.A., were used for injection of samples.

Samples

The following single components were used for calculating heats of adsorption: (1) cyclohexane, cyclohexene, benzene, *n*-hexane, 1-hexene, 1-hexyne, 4-chloro-1-butene (all reagent grade; each 20.0% (v/v) in methylene chloride); (2) *n*-pentane, 1-pentene, 1-pentyne (all reagent grade; each 20.0% (v/v) in *n*-octane).

The following mixtures were prepared for trial separation: (1) cyclohexane, cyclohexene, benzene (in *n*-decane; 1:1:1:2 by vol.); (2) *n*-hexane, 1-hexene, 1-hexyne (in methylene chloride; 1:1:1:2 by vol.); (3) *n*-pentane, 1-pentene, 1-pentyne (in *n*-octane; 1:1:1:2 by vol.).

Sample size

When samples of acetylenic or olefinic compounds were injected as the pure vapor, extremely long retention times resulted even at column temperatures well above sample boiling points, *i.e.*, b.p. + 50–70°. In addition, poor reproducibility and extreme tailing occurred. These phenomena were not observed with samples having saturated linkages. Useful chromatograms with reasonable retention times and good reproducibility were obtained for acetylenic and olefinic compounds when these were prepared in 20.0% (v/v) solutions using a sample size of 1 μl. Solvents were selected which experimentally displayed no sorption in our system and whose boiling points were compatible with our samples. For purposes of comparison, samples having saturated linkages were also prepared in an appropriate solvent as 20.0% (v/v) solutions. Initial injections of samples having unsaturated linkages exhibited a sequential decrease in retention time per injection until a constant value was obtained. This occurred only at the beginning of the day and was interpreted as due to gradual saturation of available irreversible sorption sites. Usually, three or four 1-μl injections of sample solution (20.0%) were required to "saturate" the column.

RESULTS AND DISCUSSION

Heats of adsorption

In Tables I–III are listed the heats of adsorption of the samples studied along with the respective boiling points and some pertinent operating conditions. Certain observations may be noted. In all cases, the heats of adsorption increase in the order: $3d^5$, $3d^7$, $3d^3$, *i.e.*, $MnCl_2$, $CoCl_2$, VCl_2. This is predictable in that VCl_2 possesses the greatest number of available "d" orbitals and $3d^5(MnCl_2)$ is a particularly stable state. Temperatures as high as twice the boiling point were required to elute unsaturated samples from VCl_2. It is interesting to note that the difference ΔH_a benzene $- \Delta H_a$ cyclohexane is much less on all columns than the difference ΔH_a 1-

TABLE I

HEATS OF ADSORPTION ON $MnCl_2$ IN KCAL/MOLE

Conditions as listed.

Sample	*Boiling point (°C)*	ΔH_a[a]	*Column temperature (°C)*	*Flow rate (ml/min)*
n-Pentane	36.2	2.16	60–80	16.9
1-Pentene	29.2	2.78	60–80	16.9
1-Pentyne	40.0	4.64	60–80	16.9
n-Hexane	69.0	3.24	60–80	14.8
1-Hexene	63.5	3.99	60–80	14.8
1-Hexyne	71.5	5.99	60–80	14.8
Cyclohexane	81.4	2.53	60–80	14.7
Cyclohexene	83.0	4.67	60–80	14.7
Benzene	80.1	5.29	60–80	14.7
4-Chloro-1-butene	75.0	4.61	60–80	15.3

[a] All results negative.

TABLE II

HEATS OF ADSORPTION ON $CoCl_2$ IN KCAL/MOLE

Conditions as listed.

Sample	*Boiling point (°C)*	ΔH_a[a]	*Column temperature (°C)*	*Flow rate (ml/min)*
n-Pentane	36.2	2.54	60–80	15.1
1-Pentene	29.2	3.72	60–80	15.1
1-Pentyne	40.0	7.23	60–80	15.1
n-Hexane	69.0	5.16	60–80	15.1
1-Hexene	63.5	5.96	60–80	15.1
1-Hexyne	71.5	11.87	60–80	15.1
Cyclohexane	81.4	4.04	60–80	15.7
Cyclohexene	83.0	6.53	60–80	15.7
Benzene	80.1	7.50	60–80	15.7

[a] All results negative.

pentyne — ΔH_a 1-pentene or ΔH_a 1-hexyne — ΔH_a 1-hexene, as shown in Table IV with the greatest difference noted on VCl_2. It had been predicted by MUHS AND WEISS[20] that benzene, while containing the largest amount of π-electron density, is a conjugated system and will not be as strongly chemisorbed as compounds with isolated π-bonds. This is best explained by the fact that conjugation will tend to weaken the interaction of a π-bond with a metal ion field.

For the most part, the magnitude of the heats of sorption do not compare to that which may be generally considered chemisorption (*viz.* $\leq$ 10 kcal/mole)[21] although the heats of adsorption of 1-hexyne on VCl_2 and $CoCl_2$ (13.8 and 11.87 kcal/mole, respectively) and 1-pentyne on VCl_2 (10.28 kcal/mole) are greater than this limit. However, earlier observations described under *Sample size* would indicate

TABLE III

HEATS OF ADSORPTION ON VCl_2 IN KCAL/MOLE

Conditions as listed.

Sample	*Boiling point (°C)*	ΔH_a[a]	*Column temperature (°C)*	*Flow rate (ml/min)*
n-Pentane	36.2	3.00	95–135	14.9
1-Pentene	29.2	6.02	110–135	14.9
1-Pentyne	40.0	10.28	110–135	14.9
n-Hexane	69.0	5.33	110–135	14.9
1-Hexene	63.5	7.25	110–135	14.9
1-Hexyne	71.5	13.79	100–120	14.9
Cyclohexane	81.4	4.90	110–135	14.9
Cyclohexene	83.0	7.48	110–135	14.9
Benzene	80.1	8.46	100–135	14.9
4-Chloro-1-butene	75.0	8.28	100–135	14.9

All results negative.

TABLE IV

DIFFERENCES IN HEATS OF ADSORPTION IN KCAL/MOLE[a]

ΔH_a sample A − ΔH_a sample B	$MnCl_2$	$CoCl_2$	VCl_2
ΔH_a benzene − ΔH_a cyclohexene	0.62	0.97	0.98
ΔH_a 1-pentyne − ΔH_a 1-pentene	1.86	3.51	4.26
ΔH_a 1-hexyne − ΔH_a 1-hexene	2.00	5.91	6.54

[a] All results negative.

that some physical adsorption is most certainly occurring. Recalling the observation of GARNER *et al.*[1-3], at lower concentrations, higher heats of sorption would occur for all samples except those which possess saturated linkages. Doubtless, further work is indicated to study more fully the mechanisms of the observed sorptions. This knowledge is not essential to our purpose in the work of attaining separations. However, we feel the data are indicative that chemisorption plays a role in retention of the samples studied.

Quantitative separation of mixtures

Figs. 1–3 represent the chromatograms obtained in separating a given family of samples differing primarily in the degree of π-electron density. Included are the

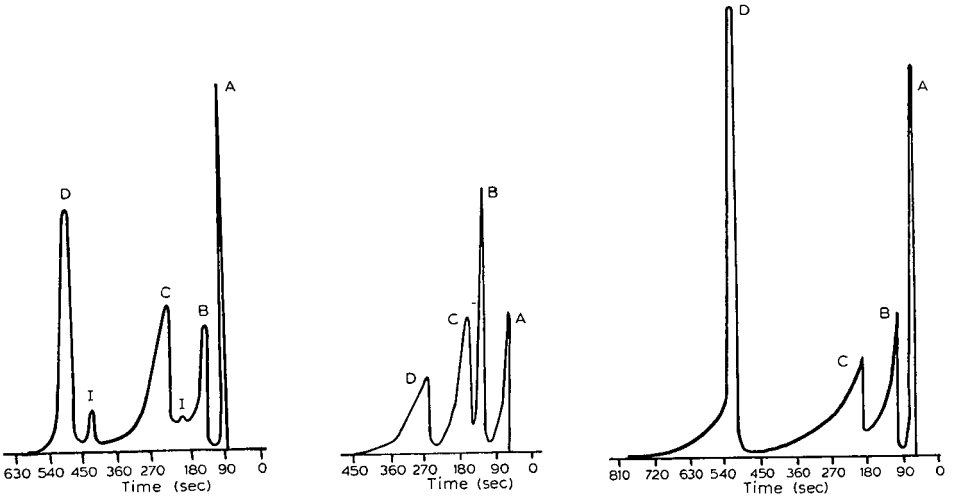

Fig. 1. Separation of *n*-pentane, 1-pentene and 1-pentyne on $CoCl_2$. Temperatures: injection 152°, detector 133°, column 29° to 230° (at 20°/min.) Flow rate: 14.7 ml/min. A = *n*-pentane, B = 1-pentene, C = 1-pentyne, D = *n*-octane, I = impurities.

Fig. 2. Separation of *n*-hexane, 1-hexene and 1-hexyne on VCl_2. Temperatures: injection 144°, detector 129°, column 26° to 190° (at 20°/min). Flow rate: 14.5 ml/min. A = Methylene chloride, B = *n*-hexane, C = 1-hexene, D = 1-hexyne.

Fig. 3. Separation of cyclohexane, cyclohexene and benzene on $MnCl_2$. Temperatures: injection 144°, detector 126°, column 75° to 190° (at 8°/min). Flow rate: 14.0 ml/min. A = cyclohexane, B = cyclohexene, C = benzene, D = *n*-decane.

TABLE V

AREAS OF PEAKS FROM FIG. 1

	Areas of peaks for C_5 hydrocarbons (cm^2)		
	n-Pentane	*1-Pentene*	*1-Pentyne*
Injection 1	336	240	682
Injection 2	250	183	520
Injection 3	319	232	652
	Ratio of peak areas relative to 1-pentene[a]		
	$A\frac{n\text{-}Pentane}{1\text{-}Pentene}$	$A\frac{1\text{-}Pentene}{1\text{-}Pentene}$	$A\frac{1\text{-}Pentyne}{1\text{-}Pentene}$
Injection 1	1.40	1.00	2.85
Injection 2	1.37	1.00	2.85
Injection 3	1.38	1.00	2.81

[a] A = area ratio.

appropriate operating conditions. In all cases, the column temperature was programmed and allowed to equilibrate for 5 min between runs. Three successive 1-μl injections were made and the resulting areas computed by triangulation. In the absence of an internal standard, in each chromatogram, the areas traced by two members of the family were divided by the area of the third member of the family in order to view reproducibility. These data are recorded in Tables V–VII. As may be noted in these tables the greatest range in peak area ratios in any series of injections was less than 3%. In addition, about 10 min are required to completely elute the samples. The data also indicate that the heat of adsorption (see Tables I–III) may be used to predict the order of elution in separation.

TABLE VI

AREAS OF PEAKS FROM FIG. 2

	Areas of peaks for C_6 hydrocarbons (cm^2)		
	n-Hexane	*1-Hexene*	*1-Hexyne*
Injection 1	443	412	423
Injection 2	485	436	448
Injection 3	490	452	468
	Ratio of peak areas relative to 1-Hexene[a]		
	$A\frac{n\text{-}Hexane}{1\text{-}Hexene}$	$A\frac{1\text{-}Hexene}{1\text{-}Hexene}$	$A\frac{1\text{-}Hexyne}{1\text{-}Hexene}$
Injection 1	1.08	1.00	1.03
Injection 2	1.11	1.00	1.03
Injection 3	1.08	1.00	1.03

[a] A = area ratio.

TABLE VII

AREAS OF PEAKS FROM FIG. 3

	Areas of peaks for cyclic compounds (cm^2)		
	Cyclohexane	*Cyclohexene*	*Benzene*
Injection 1	203	291	675
Injection 2	198	281	661
Injection 3	212	293	677
	Ratio of peak areas relative to cyclohexene[a]		
	$A\ \frac{Cyclohexane}{Cyclohexene}$	$A\ \frac{Cyclohexene}{Cyclohexene}$	$A\ \frac{Benzene}{Cyclohexene}$
Injection 1	0.698	1.00	2.32
Injection 2	0.704	1.00	2.35
Injection 3	0.713	1.00	2.31

[a] A = area ratio.

CONCLUSIONS

It is felt that the transition metal salts studied offer a practical basis for quantitative separation of *linear isomeric hydrocarbons with increasing degrees of unsaturation* (LIHIDU) and *cyclic isomeric hydrocarbons with increasing degrees of unsaturation* (CIHIDU). Compounds possessing isolated π-bonds being the more strongly adsorbed. While the data for 4-chloro-1-butene in Tables I and III are not conclusive, it is clear that no drastic difference in retention behavior can be noted in compounds having highly electronegative groups (*e.g.* halogen) not near to a π-bond. We are continuing our study of the retention behavior of compounds possessing electronegative groups more adjacent to the π-electron density along with the influence of less electronegative groups possessing non-bonding electrons (*e.g.* alcohols, ethers, etc.) as the latter may display their own interactions with the adsorbent. Additionally, we are investigating the separation of various isomers (*e.g. cis–trans* isomers). Throughout any studies, one must always beware of the influence of physical adsorption which almost always occurs regardless of the degree of chemisorption. In addition to our chromatographic studies of these systems we will be investigating the interactions of our packings with the various adsorbates under static conditions by spectroscopic techniques.

REFERENCES

1 W. D. GARNER AND S. J. VEAL, *J. Chem. Soc.*, (1935) 1487.
2 W. D. GARNER, *J. Chem. Soc.*, (1947) 1239.
3 J. J. KIPLING AND D. B. PEAKALL, *J. Chem. Soc.*, (1957) 834.
4 D. J. WHEELER, R. W. DARBY AND C. KEMBALL, *J. Chem. Soc.*, (1960) 332.
5 S. SIEGEL AND G. V. SMITH, *J. Amer. Chem. Soc.*, 82 (1960) 6087.
6 J. F. SAUVAGE, R. H. BAKER AND A. S. HUSSEY, *J. Amer. Chem. Soc.*, 82 (1960) 6090.
7 R. L. BURWELL, A. B. LITTLEWOOD, M. CORDEW, G. PASS AND C. T. H. STODDART, *J. Amer. Chem. Soc.*, 82 (1960) 6272.
8 D. J. WHEELER AND C. KEMBALL, *J. Chem. Soc.*, (1960) 1840.
9 S. SIEGEL AND G. V. SMITH, *J. Amer. Chem. Soc.*, 82 (1960) 6082.
10 A. R. BURKIN AND G. HALSEY, *Nature*, 191 (1961) 348.

11 W. R. McCLELLAN, H. H. HOEHN, N. H. CRIPPS, E. L. MUETTERTIES AND B. W. HOWK, *J. Amer. Chem. Soc.*, 83 (1961) 1601.
12 A. L. DENT AND R. J. KOHES, *J. Amer. Chem. Soc.*, 92 (1970) 1092.
13 J. J. DUFFIELD AND L. B. ROGERS, *Anal. Chem.*, 34 (1962) 1193.
14 A. G. ALTENEAU AND L. B. ROGERS, *Anal. Chem.*, 35 (1963) 915.
15 B. T. GURAN AND L. B. ROGERS, *J. Gas Chromatogr.*, 5 (1967) 574.
16 E. GIL-AV, J. HERLING AND J. SHABTAI, *J. Chromatogr.*, 1 (1958) 508.
17 J. HERLING, J. SHABTAI AND E. GIL-AV, *J. Chromatogr.*, 8 (1962) 349.
18 J. SHABTAI, J. HERLING AND J. GIL-AV, *J. Chromatogr.*, 2 (1959) 406.
19 J. SHABTAI, J. HERLING AND J. GIL-AV, *J. Chromatogr.*, 11 (1963) 32.
20 M. A. MUHS AND S. T. WEISS, *J. Amer. Chem. Soc.*, 84 (1962) 4697.
21 S. GLASSTONE AND D. LEWIS, *Elements of Physical Chemistry*, 2nd ed., Van Nostrand, Princeton, N.J., 1960, p. 560.

CHROM. 5330

THE INFLUENCE OF TEMPERATURE ON THE POLARITY OF STATIONARY PHASES USED IN GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

The present work is an attempt to assess the influence of the temperature on the polarity of the liquid phase in gas chromatography.

This effect was investigated for twenty-one liquid phases of various types of structure. It was found that the polarity depends on the temperature linearly, and its temperature coefficient can be negative, positive or zero.

It is thought, that the data obtained as a result of this work could be of practical use in gas–liquid chromatography.

INTRODUCTION

One of the most important properties of the stationary phases used in gas–liquid chromatography (GLC) is undoubtedly their polarity. As is evident from reference data however, the dipole moment of the liquid phase can not be used as a simple measure for it[1].

Some years ago ROHRSCHNEIDER[2] suggested an empirical method for the determination of the relative polarity of the liquid phase. This method, in spite of some weak points, has proved to be very satisfactory in the field of GLC.

According to this method, the polarity P could be calculated from the expression

$$P = 100 \frac{q_x - q_2}{q_1 - q_2} \quad (1)$$

where q_1, q_2 and q_x are the ratios of the specific retention volumes of butadiene–butane measured on columns coated with β,β'-oxydipropionitrile, squalane and the liquid phase under investigation, respectively.

The use of butadiene–butane as a standard pair, however, is associated with some difficulties and later authors[3], who intended to make the method more general, suggested a better pair: benzene–cyclohexane. These compounds can be found readily in every laboratory and it is possible to measure the polarity at higher temperatures with them, than with the butadiene–butane pair.

It can be assumed that the polarity calculated from eqn. 1 does not, in practice, depend on the temperature of the determination of the retention volumes, if it is one and the same for both the standard phases and for the phase under investigation.

In our earlier measurements it was observed that in some cases the polarity of the stationary phase, found according to ROHRSCHNEIDER depends on the temperature of the experiment. This effect is sometimes higher than the experimental error and must not be neglected.

The present study is an attempt to ascertain the effect of the column temperature on the gas chromatographic polarity of some stationary phases and to demonstrate the cases, in which it is necessary to take this influence into account.

EXPERIMENTAL

The necessary specific retention volumes of benzene and cyclohexane on the β,β'-oxydipropionitrile, squalane and the other stationary phases were measured with a "Fractovap" model B apparatus produced by Carlo Erba, Italy.

Pure dry nitrogen was used as carrier gas. The outlet flow rate in all experiments was 40 ml/min. The outlet pressure was atmospheric pressure, corrected for the water vapour in the soap flowmeter at its temperature. The stainless steel chromatographic column was 2 m long and had an I.D. of 4 mm. Column packing was "Sterchamol" (particle size diam. 0.2–0.3 mm) and the stationary phase consisted of 20% (w/w) of the inert support.

The measurements were carried out at intervals of 10°. The first and the last

TABLE I

SPECIFIC RETENTION VOLUMES OF BENZENE (ml)

No.	*Stationary phase*	*Temperature (°C)*							
		50	*60*	*70*	*80*	*90*	*100*	*110*	*120*
1	Squalane	177.7	133.2	102.9	77.1	61.6	47.6	38.0	31.4
2	β,β'-Oxydipropionitrile	142.9	105.3	80.3	59.2	46.7	35.6	28.1	22.9
3	Diphenyl ether	—	229.2	167.8	118.1	89.9	—	—	—
4	*m*-Bis-(*m*-phenoxyphenoxy)benzene	—	—	91.6	67.2	52.7	40.0	31.4	—
5	Glycerol	—	8.6	7.0	5.6	4.7	3.8	—	—
6	Diacetin	113.3	91.2	75.2	51.1	42.1	—	—	—
7	Triacetin	202.9	157.6	125.9	80.3	64.2	—	—	—
8	Diglycerol	11.4	9.1	7.5	6.0	5.0	—	—	—
9	Diethanolamine	28.0	24.8	22.2	17.8	—	—	—	—
10	Triethanolamine	—	39.2	29.8	21.8	17.1	13.0	—	—
11	β,β'-Iminodipropionitrile	—	99.2	74.7	54.4	42.5	32.0	—	—
12	1,2,3-Tris-(2-cyanoethoxy)-propane	—	81.6	63.0	47.1	37.6	29.1	—	—
13	1,2,3,4,5,6-Hexakis-(2-cyanoethoxy)-hexane		46.2	35.0	25.7	20.1	15.3	—	—
14	Adipic dinitrile	226.6	157.2	113.7	78.9	59.4	—	—	—
15	Butanediol-1,4	56.6	44.8	36.4	24.0	19.5	—	—	—
16	Octanediol-1,8	—	—	57.9	43.7	35.2	27.4	22.0	—
17	Decanediol-1,10	—	—	—	49.9	39.2	29.7	23.3	18.9
18	Dodecanediol-1,12	—	—	—	52.4	41.2	31.2	24.5	19.9
19	Thiodiglycol	—	38.8	30.0	22.4	17.9	—	—	—
20	Diethylene glycol	—	50.1	38.3	28.3	22.4	—	—	—
21	Triethylene glycol	—	58.6	47.5	37.5	31.2	—	—	—
22	Tetraethylene glycol	—	65.2	51.8	40.0	32.7	—	—	—
23	Polyethylene glycol 400	—	94.2	70.9	51.5	40.1	30.2	—	—

temperatures of the interval were chosen depending on the melting point and the vapour pressure of the corresponding liquid phases.

The stationary phases under investigation are shown in Table I. All of them were of commercial origin.

At present some of these stationary phases are used widely in gas chromatography, the rest were included in the work in order to show the influence of some structural changes in the molecule of the phases on the function $P = f(t^0)$.

The first measurements showed that the value of the polarity is very sensitive to the errors in the values of the retention volumes, especially when they have equal signs.

For this reason, the values of all retention volumes in the present work are the arithmetic mean of 10 consecutive measurements.

RESULTS AND DISCUSSION

The specific retention volumes of benzene and cyclohexane on the standard and investigated stationary phases at different temperatures are shown in Tables II and III.

In eqn. 1, the denominator does not depend on the stationary phase and changes only with the change of temperature. Fig. 1 shows that the difference $q_1 - q_2$ de-

TABLE II

SPECIFIC RETENTION VOLUMES OF CYCLOHEXANE (ml)

No.	*Stationary phase*	*Temperature (°C)*							
		50	*60*	*70*	*80*	*90*	*100*	*110*	*120*
1	Squalane	235.9	172.7	130.8	95.8	75.1	56.9	44.7	36.3
2	β,β'-Oxydipropionitrile	15.1	12.7	10.9	9.1	8.0	6.8	6.0	5.3
3	Diphenyl ether	—	125.3	92.3	65.4	50.0	—	—	—
4	*m*-Bis-(*m*-phenoxyphenoxy) benzene	—	—	45.9	34.5	27.7	21.5	17.2	—
5	Glycerol	—	5.7	4.3	3.1	2.4	1.8	—	—
6	Diacetin	24.6	20.8	17.9	13.3	11.4	—	—	—
7	Triacetin	46.1	38.4	32.7	23.7	20.2	—	—	—
8	Diglycerol	4.4	3.5	2.8	2.2	1.8	—	—	—
9	Diethanolamine	4.1	3.7	3.5	2.9	—	—	—	—
10	Triethanolamine	—	7.2	6.2	5.3	4.6	4.0	—	—
11	β,β'-Iminodipropionitrile	—	12.6	10.6	8.7	7.5	6.3	—	—
12	1,2,3-Tris-(2-cyanoethoxy)-propane	—	10.0	8.5	7.1	6.1	5.2	—	—
13	1,2,3,4,5,6-Hexakis-(2-cyanoethoxy)-hexane	—	6.7	5.7	4.8	4.2	3.6	—	—
14	Adipic dinitrile	29.6	23.3	18.8	14.8	12.3	—	—	—
15	Butanediol-1,4	19.0	15.3	12.6	8.5	7.2	—	—	—
16	Octanediol-1,8	—	—	28.8	22.5	18.6	15.0	12.4	—
17	Decanediol-1,10	—	—	—	29.6	23.2	17.5	13.7	11.1
18	Dodecanediol-1,12	—	—	—	32.2	25.6	19.7	15.7	12.9
19	Thiodiglycol	—	6.8	6.0	5.3	4.8	—	—	—
20	Diethyleneglycol	—	10.1	8.0	6.2	5.0	—	—	—
21	Triethylene glycol	—	11.0	10.0	9.0	8.3	—	—	—
22	Tetraethylene glycol	—	12.7	11.3	9.9	8.9	—	—	—
23	Polyethylene glycol	—	16.3	14.3	12.5	11.2	9.8	—	—

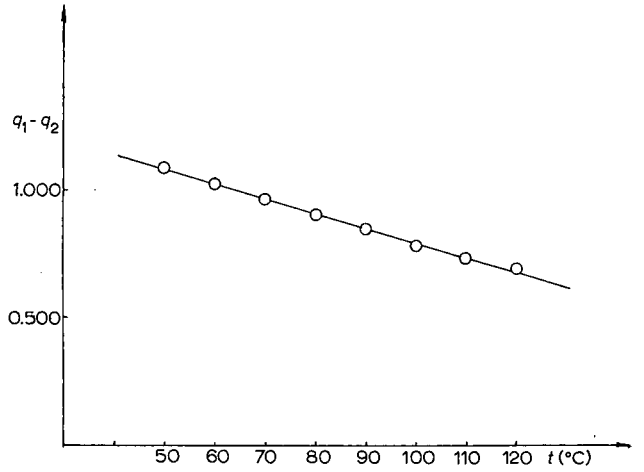

Fig. 1. Plot of the difference q_1-q_2 (eqn. 1) *vs.* temperature.

creases linearly when the temperature increases. It is evident, that the polarity P according to eqn. 1 will be independent of temperature only if the numerator changes in the same way. The data for the polarities at different temperatures (Table III) show that this condition is only fulfilled in a few cases.

TABLE III

POLARITIES OF THE LIQUID PHASES AT DIFFERENT TEMPERATURES

No.	*Stationary phase*	*Temperatures (°C)*							
		50	*60*	*70*	*80*	*90*	*100*	*110*	*120*
1	Squalane	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	β,β′-Oxydipropionitrile	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3	Diphenyl ether	—	36.5	37.4	39.0	40.0	—	—	—
4	*m*-Bis-(*m*-phenoxyphenoxy)-benzene	—	—	41.4	42.3	43.0	43.6	44.8	—
5	Glycerol	—	28.6	33.0	39.0	44.1	50.7	—	—
6	Diacetin	71.7	73.3	74.8	75.1	76.4	—	—	—
7	Triacetin	69.9	70.4	70.9	69.0	69.0	—	—	—
8	Diglycerol	48.8	51.9	54.8	59.2	62.7	—	—	—
9	Diethanolamine	87.3	90.6	93.8	96.9	—	—	—	—
10	Triethanolamine	—	82.2	80.7	78.8	77.0	74.7	—	—
11	β,β′-Iminodipropionitrile	—	98.0	98.0	98.2	98.3	98.5	—	—
12	1,2,3-Tris-(2-cyanoethoxy)-propane	—	99.3	100.2	101.5	102.7	104.2	—	—
13	1,2,3,4,5,6-Hexakis-(2-cyanoethoxy)-hexane	—	92.2	91.5	90.7	89.9	88.9	—	—
14	Adipic dinitrile	91.8	91.5	91.1	90.7	90.3	—	—	—
15	Butanediol-1,4	54.5	56.4	58.2	60.4	61.2	—	—	—
16	Octanediol-1,8	—	—	41.8	42.3	42.5	42.7	43.2	—
17	Decanediol-1,10	—	—	—	35.8	36.8	38.3	40.5	42.2
18	Dodecanediol-1,12	—	—	—	35.8	34.3	34.6	35.5	36.1
19	Thiodiglycol	—	84.6	82.3	79.6	77.1	—	—	—
20	Diethylene glycol	—	78.3	80.6	83.6	86.2	—	—	—
21	Triethylene glycol	—	81.3	80.2	78.8	77.4	—	—	—
22	Tetraethylene glycol	—	79.8	78.7	77.6	76.5	—	—	—
23	Polyethylene glycol	—	84.9	82.0	78.5	75.3	71.0	—	—

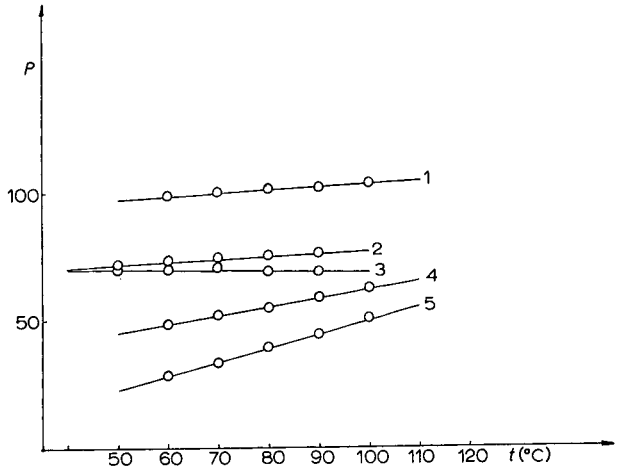

Fig. 2. Dependence of the polarity of the liquid phase on the temperature. 1 = 1,2,3-tris-(2-cyanoethoxy)-propane; 2-diacetin; 3 = triacetin; 4 = diglycerol; 5 = glycerol.

From a formal view-point, the phases under investigation could be divided into three groups.

In the first group are the phases where the increase of the temperature leads to an increase in the polarity, *e.g.* $\Delta P/\Delta t > 0$. These are phases such as diethylene glycol; butanediol-1,4; decanediol-1,10; 1,2,3-tris-(2-cyanoethoxy)-propane; diethanolamine; glycerol; diglycerol; diacetin; diphenyl ether and *m*-bis-(*m*-phenoxyphenoxy)-benzene.

The second group includes the liquid phases 1,2,3,4,5,6-hexakis-(2-cyanoethoxy)-hexane; triethylene glycol; tetraethylene glycol; polyethylene glycol 400; thiodi-

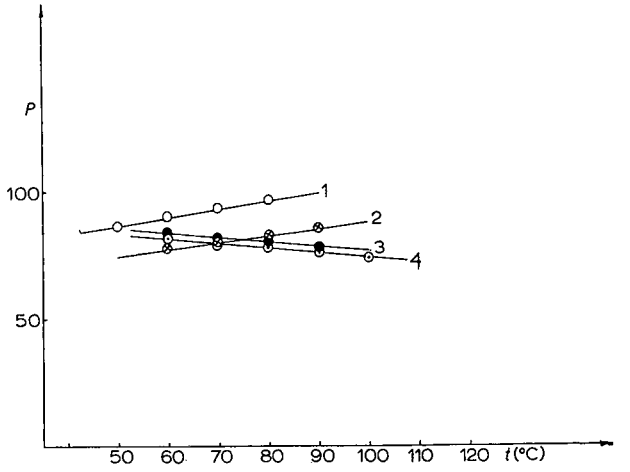

Fig. 3. Polarity of the liquid phases depending on the temperature. 1 = diethanolamine; 2 = diethylene glycol; 3 = thiodiglycol; 4 = triethanolamine.

glycol and triethanolamine. They decrease the polarity when the temperature increases *e.g.* $\Delta P/\Delta t < 0$.

The third group includes those phases whose polarity is practically temperature independent, $\Delta P/\Delta t = 0$, for example β,β'-iminodipropionitrile; triacetin; adipic-dinitrile; octanediol-1,8 and dodecanediol-1,12.

Fig. 2 presents graphically the relationship $P = f(t^0)$ for glycerol and other phases, which are glycerol derivatives. Acetylation or cyanoethylation of the glycerol hydroxy groups leads to a decrease in the slope of the lines. For instance $\Delta P/\Delta t$ is about +0.55 units/degree for the glycerol, while the same quantity is +0.10 and +0.13 units/degree for diacetin and 1,2,3-tris-(2-cyanoethoxy)-propane. In the case of triacetin the slope of the line becomes negative (−0.05 units/degree).

In addition, diglycerol has a lower temperature coefficient than glycerol. In this case the total number of the hydroxy groups, as compared with glycerol, is higher but their percentage per unit molecular weight is lower. On the other hand in the diglycerol molecule there is a new structural element—the ether bond.

The dependence between polarity and temperature for diethanolamine, diethylene glycol, thiodiglycol and triethanolamine is shown in Fig. 3. The common

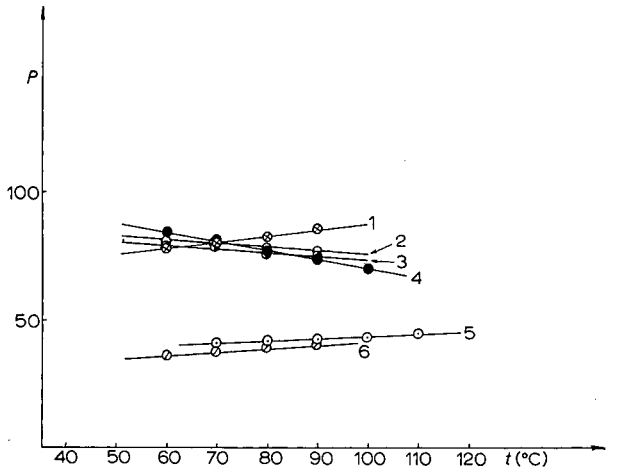

Fig. 4. Plot of the polarity of the liquid phases *vs.* temperature. 1 = diethyleneglycol; 2 = triethylene glycol; 3 = tetraethylene glycol; 4 = polyethylene glycol 400; 5 = *m*-bis-(*m*-phenoxyphenoxy)-benzene; 6 = diphenyl ether.

feature in the structure of these liquid compounds is the presence of 2-hydroxyethyl groups. The first three of them differ only in the heteroatom in the chain.

Examination of the functions $P = f(t^0)$ in Fig. 3 shows that diethylene glycol and diethanolamine have almost equal positive slopes while thiodiglycol and triethanolamine have almost equal but negative slopes. A very interesting fact is the great difference in the temperature coefficient of polarity in the case of thiodiglycol as compared with diethylene glycol and diethanolamine.

Fig. 4 shows the graphical presentation of the changes in the polarity of some ethylene glycols with change of temperature. It can be seen that the highest temper-

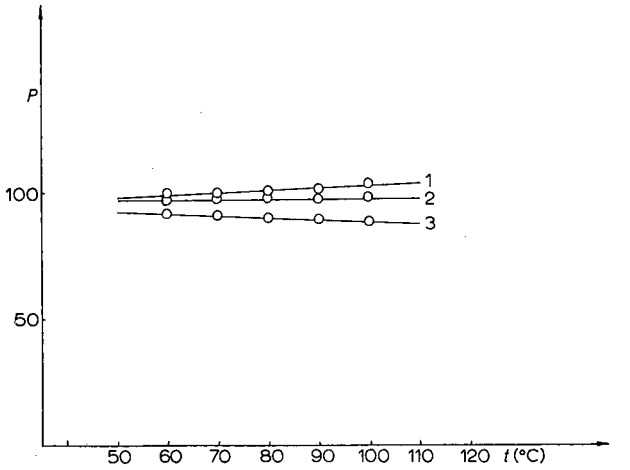

Fig. 5. Polarity of the liquid phases as a function of temperature. 1 = 1,2,3-tris-(2-cyanoethoxy)-propane; 2 = β,β'-iminodipropionitrile; 3 = 1,2,3,4,5,6-hexakis-(2-cyanoethoxy)-hexane.

ature coefficient of polarity (positive value) is shown by diethylene glycol. The increase in the number of $-OCH_2CH_2-$ groups leads to a decrease in the slopes (already negative) of triethylene and tetraethylene glycols and polyethylene glycol 400. The polyethylene glycol-400 line has the most negative slope. Data concerning diphenyl ether and *m*-bis-(*m*-phenoxyphenoxy)-benzene are shown in the same figure. The line for diphenyl ether has a higher positive temperature coefficient of polarity than the second liquid phase, but the difference is not very great.

The data concerning liquid phases with propionitrile groups are given in Fig. 5. It can be seen that β,β'-iminodipropionitrile has a practically constant polarity and it has a value close to the value of the standard β,β'-oxydipropionitrile (P = 100).

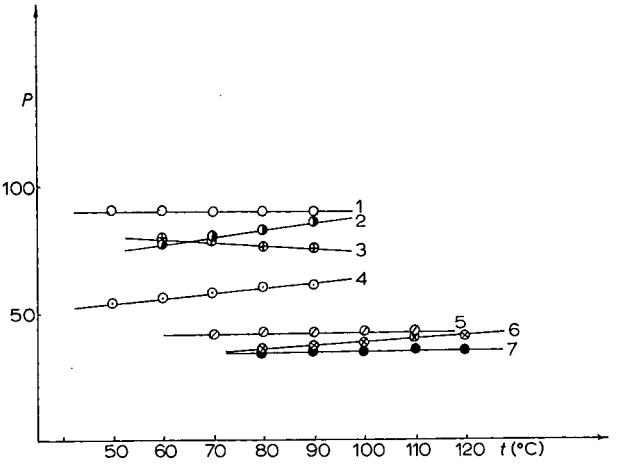

Fig. 6. Dependence of the polarity of the liquid phases on the temperature. 1 = Adipic dinitrile; 2 = diethylene glycol; 3 = tetraethylene glycol; 4 = butanediol-1,4; 5 = octanediol-1,8; 6 = decanediol-1,10; 7 = dodecanediol-1,12.

This means that the substitution of an imino group by an ether bond does not influence the temperature coefficient $\Delta P/\Delta t$.

An increase in the number of the propionitrile groups in the molecule of the phase decreases the slope of the line $P = f(t^0)$ from a positive to a negative value (*e.g.* see 1,2,3-tris-(2-cyanoethoxy)-propane and 1,2,3,4,5,6-hexakis-(2-cyanoethoxy)-hexane).

Study of the influence of the temperature on the polarities of phases from the group of alkanediols (Fig. 6) shows that butanediol-1,4 and decanediol-1,10 have almost equal positive temperature coefficients, +0.17 and +0.16 units/degree, respectively.

Octanediol-1,8 and dodecanediol-1,12 are similar in their behavior too, but differ from the first two diols. It is now difficult to explain why there is no regular change of the slope of $P = f(t^0)$ for all diols.

The presence of an extra ether bond in diethylene glycol as compared with butanediol-1,4 hardly changes the slope of the line (Fig. 6); however the accumulation of several ether bonds (*e.g.* tetraethylene glycol as compared with octanediol-1,8) leads to a considerable difference. In this case tetraethylene glycol has the same number –OH and $-CH_2-$ groups as octanediol-1,8, but has three –O– bonds more.

The exchange of both hydroxy groups in butanediol-1,4 with nitrile groups in adipic dinitrile makes the polarity of the liquid phase almost independent of temperature.

In conclusion it might be summarized that:

(1) Polarity of the liquid phase depends linearily on the temperature.

(2) The dependence between the polarity and temperature can have a positive, negative or zero temperature coefficient.

(3) In a given temperature interval one liquid phase can be more polar than another phase, but they can exchange their polarities in another temperature interval.

REFERENCES

1 H. Rotzsche and M. Hofmann, in E. Leibnitz and H. G. Struppe (Editors), *Handbuch der Gas-Chromatography*, Verlag Chemie, Weinheim/Bergstr., 1967, p. 351.
2 L. Rohrschneider, *Z. Anal. Chem.*, 170 (1959) 256.
3 P. Chovin and J. Lebbe, *Communication aux Journées International d'Étude des Méthodes de Séparation Immédiate et de Chromatographie, juin 1961, Paris.*

CHROM. 5368

GAS CHROMATOGRAPHIC ANALYSIS OF PYRROLIC ACID ESTERS FROM THE POTASSIUM PERMANGANATE OXIDATION OF BILE PIGMENTS

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SUMMARY

The NICOLAUS permanganate degradation of bile pigments and porphyrins has been adapted for use with gas chromatography in an attempt to improve the sensitivity of the method and to make it quantitative. Gas–liquid chromatographic systems for the separation of the ethyl esters of 3-methylpyrrole-2,4,5-tricarboxylic acid and 4-carboxyethyl-3-methylpyrrole-2,5-dicarboxylic acid are described. These systems have been used to estimate the content of non-IXα isomer in ox gallstone bilirubin, which is calculated to be 1–3% of the total if the non-IXα fraction is assumed to be wholly γ isomer or 2–6% if it is a β or δ isomer.

INTRODUCTION

The four isomers of biliverdin derived from the protoporphyrin ring by fission of the α, β, γ and δ bridges are shown in Fig. 1. The predominantly IXα structure assumed for natural bile pigments on the basis of early work was confirmed by GRAY *et al.*[1] using the method developed by NICOLAUS[2] for the paper chromatographic identification of pyrrole carboxylic acids formed on oxidation of naturally occurring polypyrrole pigments. Bile pigment central rings yield pyrrole 2,5-carboxylic acids on potassium permanganate oxidation. IXα isomers of biliverdin and bilirubin give 4-carboxyethyl-3-methyl-pyrrole-2,5-dicarboxylic acid (I), γ isomers 3-methylpyrrole-2,4,5-tricarboxylic acid (II). β and δ isomers and mixtures of isomers give both. This paper describes an attempt to make this technique quantitative and to improve its sensitivity by adapting it to gas–liquid chromatography (GLC). The oxidation and extraction procedure described by NICOLAUS has been slightly modified, since the visual assessment of a suitable excess of potassium permanganate gave very variable yields. Preliminary experiments were therefore directed to determining the optimum proportion of oxidant and the optimum oxidation time. This gave no markedly improved yields of pyrrolic acids, but allowed definition of a standard oxidation procedure giving reliable yields. Precipitation of oxalate in the extraction procedure has been omitted because it does not much improve the final chromatogram

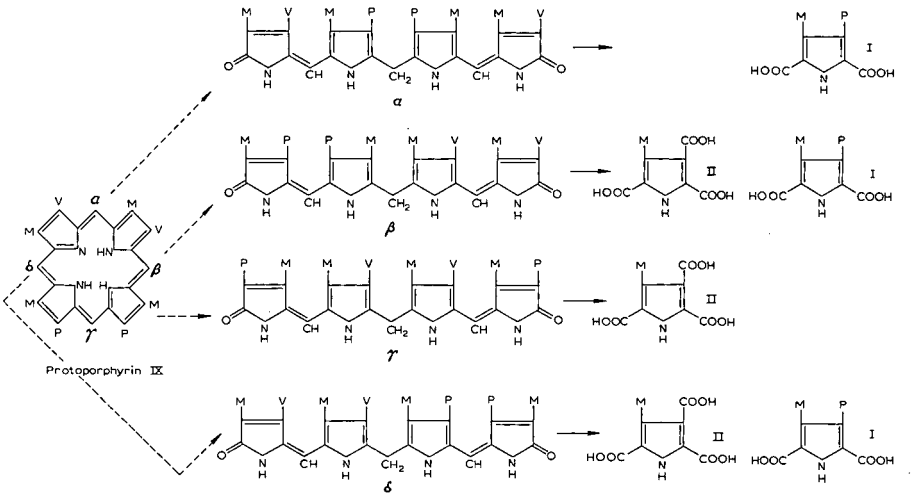

Fig. 1. The four isomeric forms of bilirubin IX and their NICOLAUS degradation products.

and causes loss of the pyrrolic acids. Pyrrolic acids decompose on melting necessitating formation of derivatives for GC. The ethyl esters are convenient because the penultimate step in pyrrolic acid synthesis is the formation of a partial ethyl ester. Final hydrolysis is wasteful causing polymerisation of the free pyrrolic acids and the partial esters are more stable for storage. The method of final esterification must be applicable to imprecisely known quantities of free pyrrolic acids in bile pigment oxidation mixtures. Of diazoethane, 5% ethanolic sulphuric acid and ethanolic boron trifluoride, the last two esterification reagents were unsuitable, causing polymerisation of free pyrrolic acids. Pyrrolic acid (II) in a moderate excess of diazoethane formed the triester triethyl-3-methylpyrrole-2,4,5-tricarboxylate (III); a larger excess esterified the imino hydrogen as well forming triethyl-1-ethyl-3-methylpyrrole-2,4,5-tricarboxylate (IV). The imino hydrogen of (I) was unaffected by large excesses of diazoethane, only diethyl-4(2)-carboxyethyl-ethyl-3-methylpyrrole-2,5-dicarboxylate (V) being formed. The method developed for the separation and identification of (III), (IV) and (V) has been used to estimate the proportion of non-IXα isomer in natural (ox gallstone) bilirubin.

MATERIALS AND METHODS

Chemicals

Ox gallstone bilirubin and protoporphyrin were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Haemin was prepared from whole human blood by the method of NASHIDA AND LABBE[3].

The pyrrolic acid esters (III), (IV) and (V) for use as GLC standard were prepared from the diethyl ester of (II) 3,5-diethoxycarbonyl-4-methylpyrrole-2-carboxylic acid and the monoethyl ester of (I) 3(2-carboxyethyl)-5-ethoxycarbonyl-3-methylpyrrole-2-carboxylic acid. The diester of (II) was prepared by reacting ethyl aceto-acetate with glacial acetic acid and sodium nitrite and condensing the resulting

oxime with ethyl oxaloacetate[4]. The monoester of (I) is available by the route described by FISCHER AND ORTH[5]. We are grateful to Dr. R. A. NICOLAUS and to Dr. A. H. JACKSON for samples of this compound. (IV) and (V) were prepared by treating the diester of (II) and the monoester of (I) with a large excess of diazoethane. (III) was prepared by treatment of the diester of (II) with 5% ethanolic sulphuric acid for 24 h. The crude preparations of (III), (IV) and (V) were purified on 25 ml columns of silicic acid, conditioned at 110° for 24 h before use. The columns were made up in light petroleum (b.p. 40–60°) and washed with 50 ml of light petroleum before application of the samples in the minimum quantity of methanol. The pyrrolic acid esters were eluted from the columns as the proportion of ether in the column washings was increased. The emergent fractions were scanned for pyrrolic acid ester content by measuring the absorption spectra from 200–400 mμ in a Unicam SP. 800 UV spectrophotometer. Fractions so located were then further scanned by GLC and chromatographically pure fractions were retained for use as standards. The melting points and C, H and N analysis values of the standard preparations of pyrrolic acid esters are given in Table I.

TABLE I

THE MELTING POINTS, AND C, H AND N ANALYSIS VALUES OF THE STANDARD PYRROLIC ACID ESTER PREPARATIONS

Pyrrolic acid ester	*Description*	*Melting point (°C)*	*UV maxima (mμ)*	*C%*		*H%*		*N%*	
				Observed	*Theoretical*	*Observed*	*Theoretical*	*Observed*	*Theoretical*
III	white solid	140	222 275	55.3	56.6	6.4	6.3	—	4.7
IV	white oil	—	225 260	59.2	59.0	7.1	7.0	4.4	4.3
V	white solid	185	222 277	58.8	59.0	7.2	7.0	4.2	4.3

Storage of standard solutions of pyrrolic acid esters

To avoid the error and wastage inherent in repeatedly weighing small quantities of standard compounds, a solution was prepared and divided into small aliquots which were dried under a current of nitrogen and stored at 0° for use as required. 2.8 mg, 2.6 mg and 3.3 mg of (III), (IV) and (V), respectively were transferred to a 5 ml volumetric flask and dissolved in ethanol (spectroscopic grade: Hopkin and Williams Ltd.). 100 μl portions of this solution were distributed into 48 acid washed vials, dried and stored at 0°.

Preparation of a calibration curve

100 μl of spectroscopic ethanol or chloroform was added to one of the vials containing known quantities of the three standard solutions giving a solution containing 0.56, 0.52 and 0.66 μg/μl of (III), (IV) and (V), respectively. The vial was closed immediately with a rubber injection septum to minimise evaporation. The detector responses in mm^2 at noted attentuation and chart speed were measured for

1, 2, 5 and 10 μl quantities of this solution; all measurements were made with 1 and 10 μl Hamilton microsyringes.

Potassium permanganate oxidation of bile pigments and porphyrins

The oxidation and extraction procedure described by NICOLAUS was modified slightly since errors in determining a suitable excess of potassium permanganate visually allowed considerable variation in the quantity of oxidant added. Theoretically, 22 and 19 oxygen atoms, respectively are required to degrade one molecule of protoporphyrin or bilirubin to four pyrrole rings with the vinyl side chains oxidised to carboxyl groups, so that one mole of protoporphyrin would require $22 \times \frac{2}{3}$ moles of alkaline potassium permanganate, and one mole of bilirubin $19 \times \frac{2}{3}$ moles of alkaline potassium permanganate. The optimum proportion of oxidant was found to be 2 molar equivalents of alkaline potassium permanganate. For 1 mg of protoporphyrin or 1 mg of bilirubin 0.113 ml saturated potassium permanganate and 0.107 ml saturated potassium permanganate, respectively are required since saturated potassium permanganate contains 6.3 g/100 ml at 20°. The optimum oxidation time was between 2 and 5 min and the yield was improved if ten times the volume of 1 *N* potassium carbonate recommended by NICOLAUS is used.

Samples of pigment from 10–100 mg were dissolved in 2–20 ml of 1 *N* potassium carbonate and two molar equivalents of saturated potassium permanganate (as previously defined) added fairly rapidly and dropwise to the well-shaken solution. After 5 min excess oxidant was destroyed with a minimum quantity of sodium *meta*-bisulphite and manganese dioxide removed by filtration. The precipitate and the reaction vessel were rinsed with 2–20 ml of hot distilled water and the washings added to the filtrate. The cooled filtrate was washed with 2 × (5–20) ml of peroxide-free ether before acidification and extraction into 5 × (5–20) ml of ether. The extract was reduced to 10 ml before esterification.

The esterification of pyrrolic acid extracts with diazoethane

Freshly made diazoethane was added to the ether extracts of the pyrrolic acids until there was a visible excess. The extracts were left in the dark at 4° for 16 h. Excess diazoethane was removed by the addition of a few drops of glacial acetic acid. The extracts were washed with sodium hydrogen carbonate and then with distilled water, evaporated in a current of air and dried in a vacuum desiccator.

Preparation of columns and column packings

Diatoport S 60–80 mesh as supplied by Hewlett Packard was impregnated with stationary liquid by the method of PARCHER AND URONE[6]. Newly packed columns were conditioned at 250° for about 64 h before use.

RESULTS AND DISCUSSION

The choice of GLC conditions for the analysis of pyrrolic acid esters

The Pye Panchromatograph with argon ionization detector was used in preliminary work. Samples were evaporated onto stainless steel gauzes for application to the columns. In later work using a Pye 104 gas chromatograph with flame ionization detector samples were applied by liquid injection. Acid washed Chromosorb P was

used for column packings with a high percentage of stationary phase, and silanised Diatoport S for columns with a low loading of stationary phase. A moderate mesh size (60–80) has been used for all columns.

The use of four stationary liquids has been investigated. These were Apiezon L (APL); the methyl silicone gum SE-30; poly(diethylene glycol succinate) (DEGS); and poly(neopentyl glycol adipate) (NGA). Samples of (III), (IV) and (V) were run on 5 ft. × ¼ in. 10% columns at temperatures from 150° to 225° at argon flow rate 100 ml/min. The two non-polar columns were unsatisfactory, giving poor resolution and badly tailing peaks, but on the polar columns the compounds were well separated with symmetrical peaks. Initially 5 ft. × ¼ in. columns of 1% NGA and DEGS on Diatoport S, 60–80 mesh were used at 200° and flow rates of 100 ml/min, for argon on the Panchromatograph, and 50/50/500, for argon, hydrogen and air, respectively on the Pye 104. These were adequate for the identification of (V) in the oxidation product of 1–2 mg of bilirubin and of (III), (IV) and (V) from 3–4 mg of protoporphyrin. The oxidation of 50 mg of pigment provides sufficient material for 10 GLC samples. Modifications of this system were necessary to investigate the non-IXα content of natural bilirubin since the pyrrolic acids are only minor products of the permanganate oxidation, and greater resolution is required if the area of the chromatogram where ester IV emerges is not to be obscured at high sensitivities by tailing early-emerging components. The most effective isothermal system was a 15 ft. × ¼ in. column at 200° with flow rates of 15/15/500 for argon, hydrogen and air, respectively. Resolution was further increased by temperature programming. The best resolution of (IV) from esterified oxidation extracts was obtained on 15-ft. columns of 1% NGA and DEGS programmed from 150 to 200° at 1°/min with flow rates

TABLE II

THE RETENTION TIMES OF (III), (IV) AND (V) UNDER VARIOUS CONDITIONS OF GLC

Retention time (min)			*Temperature (°C)*	*Column*	*Argon flow rate (ml/min)*
III	*IV*	*V*			
9.5	6.8	14	195	1% NGA	50
3.1	2.3	4.4	215	5 ft.	
44	27	60	195	10% NGA	100
22	15	32	210	5 ft.	
14	11	21	220		
40	29	60	194	1% NGA	15
13	9.9	19	220	15 ft.	
18	13	30	197	1% NGA	50
7.2	5.3	10	220	15 ft.	
7.9	4.4	10	195	1% DEGS	50
2.9	1.7	3.6	215	5 ft.	
21	12	27	190	10% DEGS	100
13	7.7	16	204	5 ft.	
6.7	4.1	8.3	222		
35	19	45	193	1% DEGS	15
11	6.4	13	220	15 ft.	
14	7.6	18	195	1% DEGS	50
4.8	2.9	5.9	220	15 ft.	

15/15/500 for argon, hydrogen and air, respectively. The retention times of (III), (IV) and (V) under various conditions of GLC are summarised in Table II.

The qualitative identification of the pyrrolic acid esters

Identification of the pyrrolic acid esters were based wherever sample size would permit, on comparison with the retention times of standard pyrrolic acid esters run under the same conditions on both NGA and DEGS. It was usually simple to identify (V), the product of the α isomers of bile pigments. To identify very small peaks of (IV) and (III) in esterified oxidation extracts from natural bilirubin, samples of mixed standards and unknowns were run to see if the tentatively identified peaks emerged with the standard compounds. To minimise the risk of misidentification (III) and (IV) were identified by their retentions relative to (V) on DEGS and NGA. The ratio of the adjusted retention times of any two solutes, *i.e.* the intervals between the emergence of an unabsorbed gas and the solutes, depends only on the operating temperature and the stationary phase. Table III records the retentions of (III) and (IV) relative to (V) for the conditions used.

TABLE III

THE GLC RETENTIONS OF (III) AND (IV) RELATIVE TO (V)

Stationary liquid	*Temperature (°C)*	*Retention time of (IV)/retention time of (V)*			*Retention time of (III)/retention time of (V)*		
		No. of observations	*Mean value*	*Standard deviation*	*No. of observations*	*Mean value*	*Standard deviation*
NGA	195	9	0.67	0.93×10^{-2}	9	0.47	1.0×10^{-2}
	198	19	0.67	0.4×10^{-2}	19	0.48	1.0×10^{-2}
	200	22	0.67	0.67×10^{-2}	22	0.48	0.58×10^{-2}
	150–200 at 1°/min	7	0.78	0.85×10^{-2}	7	0.64	0.9×10^{-2}
DEGS	195	15	0.77	0.71×10^{-2}	15	0.42	0.71×10^{-2}
	198	4	0.78	0.5×10^{-2}	4	0.43	0.5×10^{-2}
	200	26	0.78	0.8×10^{-2}	26	0.44	1.3×10^{-2}
	150–200 at 1°/min	5	0.85	0.9×10^{-2}	5	0.63	1.3×10^{-2}

Quantitative use of the GLC method

Calibration curves were prepared before each series of oxidations. From this data the micromolar response factors for (III), (IV) and (V) at a standard attenuation (y) and chart speed (t) may be determined as follows:

$$\mu F_r = \frac{\text{sample size } (\mu\text{moles})}{\text{observed peak area}} \times \frac{y't}{yt'}$$

when y' and t' are the attentuation and chart speed at which the peak area was measured. The quantity of material in an unknown sample is determined by multiplying the observed peak area, corrected to attentuation y and chart speed t, by the response factor. To determine the proportion of isomers in a sample of bile pigment the response factors relative to the response factor for (V) were calculated. The relative molar response factors (f_{III}, f_{IV} and f_V) are therefore:

$f_{III} = \mu F_{rIII}/\mu F_{rV}$
$f_{IV} = \mu F_{rIV}/\mu F_{rV}$
$f_V = 1$

The response of the flame ionization detector to four different quantities of a standard solution of the pyrrolic acid esters (III), (IV) and (V) was measured on thirteen different occasions under different GLC conditions. Visual inspection of the values obtained from these data suggested that, despite the use of different sample quantities, changes in the chromatographic conditions and daily variations of the equipment, f_{III}, f_{IV} and f_V were sufficiently close for the mean values to be used in calculating analytical results without continuous calibration of the detector response. This was formally tested using the statistical analysis of variance model. The method adopted was to determine whether a statistically significant proportion of the total variation in the values for the relative molar response factors originated from daily variation or from variation in sample size. If the hypothesis that (a) sample size and (b) day and conditions of the experiment have no effect on results is true, then the calculated statistic has an "F" distribution so that, with the choice of an appropriate level of significance (5% and 2.5%), the hypothesis is rejected if the calculated statistic is greater than the tabulated value for the cumulative "F" distribution with the relevant degree of freedom. However, if these tests show that the hypotheses cannot be rejected then it can be concluded that the mean values of the response factors can be used in calculations. Alternatively, if the hypotheses are rejected then it must be concluded that owing to variation in sample size and/or conditions of use the chromatograph will have to be calibrated before each series of analyses.

The hypotheses under test may be formally expressed as:

1st. null hypothesis (H_0^1). The effect of the different sample sizes is not significant. Hypothesis to be accepted if the estimated variance ratio (F^1) with 3 and 36 degrees of freedom $\leqslant F_{5\%}$ and $F_{2.5\%}$ (3,36).

2nd. null hypothesis (H_0^2). The effect of the day and/or condition of the experiment is not significant. Hypothesis to be accepted if the estimated variance ratio (F^2) with 12 and 36 degrees of freedom $\leqslant F_{5\%}$ and $F_{2.5\%}$ (12,36)

	5%	2.5%
F (3,36)	2.87	3.51
F (12,36)	2.03	2.33

Results. (1) For $f_{III} - F^1 = 0.529 < F_{5\%}$ (3,36) therefore accept H_0^1; for $f_{IV} - F^1 = 1.18 < F_{5\%}$ (3,36) therefore accept H_0^1. (2) For $f_{III} - F^2 = 14.5 < F_{2.5\%}$ (12,36) therefore reject H_0^2; for $f_{IV} - F^2 = 12.55 < F_{2.5\%}$ (12,36) therefore reject H_0^2. For both F_{III} and f_{IV} the variation in quantities did not significantly affect the results at the 5% and 2.5% significance levels, whereas the occasion and/or GLC conditions when the readings were taken did significantly affect the results. This means that although the detector response to varying quantities of each of the pyrrolic acid esters is linear (on thirteen occasions for the range observed), the ratios between the gradients of the calibration curves of (III), (IV) and (V) vary with day and/or GLC conditions so that to estimate the proportions of pyrrolic acid esters in a sample the detector must be recalibrated before each series of analyses. The relative molar response factors are also available from the gradients m_{III}, m_{IV} and m_V of the

TABLE IV

THE MEAN VALUES OBTAINED FOR THE RELATIVE MOLAR RESPONSE FACTORS OF (III) AND (IV) ON THIRTEEN SEPARATE OCCASIONS UNDER VARIOUS GLC CONDITIONS

Column	*Argon flow rate*	*Temperature (°C)*	f_{III}	f_{IV}
1% NGA 5 ft.	50 ml/min	200	1.0725	1.0025
1% NGA 5 ft.	50 ml/min	200	1.0825	1.0125
1% NGA 5 ft.	15 ml/min	200	1.0275	0.88
1% NGA 15 ft.	15 ml/min	200	1.0575	1.005
1% NGA 15 ft.	15 ml/min	200	1.0325	1.9925
1% DEGS 5 ft.	50 ml/min	200	1.0675	1.9825
1% DEGS 5 ft.	50 ml/min	200	1.055	0.9975
1% DEGS 5 ft.	50 ml/min	200	1.0675	0.9875
1% DEGS 5 ft.	15 ml/min	200	1.065	0.9025
1% DEGS 15 ft.	15 ml/min	200	1.0625	0.9025
1% DEGS 15 ft.	15 ml/min	200	1.0725	1.0
1% NGA 15 ft.	15 ml/min	150–200 at 1°/min	1.155	0.98
1% DEGS 15 ft.	15 ml/min	150–200 at 1°/min	1.0625	0.9875

calibration curves ($y' = m'x'$ when y' is the observed peak area and x' is the sample concentration in moles) of (III), (IV) and (V), respectively.

$$f_{III} = m_V/m_{III} \qquad f_{IV} = m_V/m_{IV}$$

However for calculations in the present work the appropriate values for f_{III} and f_{IV} are estimated as the arithmetic means of the values obtained from four different quantities of the standard solution of (III), (IV) and (V), on the relevant day. These values are collected in Table IV.

The percentage molar composition of a sample is calculated from the relevant relative response factors and observed peak areas in the following way. If a portion of sample containing a moles of (III), b moles of (IV) and c moles of (V) gives peak areas A, B and C mm² at the same attenuation and chart speed for (III), (IV) and (V), respectively, then:

$$a:b:c = Af_{III}:Bf_{IV}:C$$

and the sample therefore contains:

$$100Af_{III}/(Af_{III} + Bf_{IV} + C)\% \text{ of (III)}$$
$$100Bf_{IV}/(Af_{III} + Bf_{IV} + C)\% \text{ of (IV)}$$
$$100C/(Af_{III} + Bf_{IV} + C)\% \text{ of (V)}$$

The relative stabilities of the pyrrolic acids to the oxidation, esterification and extraction procedure

In order to calculate the proportion of isomers present in a sample of bile pigment from a chromatogram of the esterified oxidation products, the relative stabilities of the pyrrolic acids and esters to the oxidation, esterification and extraction procedure must be known. Since it has not been possible to synthesise 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid, the precursor of (II), direct recovery measurements have not been possible. The relative stabilities of the pyrrolic acids have therefore been deduced from an examination of the oxidation products of a ring compound such as protoporphyrin, haemin or haematin, assuming that permanganate oxidation of these compounds result in random ring fission yielding equal quantities of 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid and (I). On oxidation and esterification with excess diazoethane, therefore, $2k$ moles of haemin will yield, $r_{IV}k/100$ moles of (IV) and $r_V k/100$ moles of (V), if r_{IV} and r_V are the overall percentage molar recoveries of (IV) and (V). If a portion of an esterified oxidation extract gives peak areas B_1 and C_1 mm² at constant attentuation and chart speed for (IV) and (V), then

$$r_{IV}k/100 : r_V k/100 = B_1 f_{IV} : C_1$$

if f_{IV} is the relevant response factor.

TABLE V

THE VALUES OBTAINED FOR K, THE FACTOR FOR THE RECOVERY OF PYRROLIC ACID ESTER (IV) RELATIVE TO (V)

No.	*Starting material*	*GLC conditions*	B_I	C_I	f_{IV}	K
1	haematin	15 ft. 1% NGA 150–200 at 1°/min.	360	840	0.98	0.42
2	haematin	see (1)	125	514	0.98	0.43
3	haematin	see (1)	55	121	0.98	0.45
4	haematin	15 ft. 1% DEGS 150–200 at 1°/min	156	352	0.9875	0.44
5	haematin	see (4)	108	237	0.9875	0.45
6	protoporphyrin	15 ft. 1% DEGS 200°	108	2000	0.9975	0.205
7	protoporphyrin	see (6)	725	1680	0.98	0.425
8	protoporphyrin	5 ft. 1% NGA 200°	540	1260	0.99	0.43
9	protoporphyrin	5 ft. 1% DEGS 200°	505	1060	0.9875	0.47
10	protoporphyrin	15 ft. 1% DEGS 200°	116	267	0.99	0.43
11	haemin	15 ft. 1% NGA 200°	232	520	1.005	0.45
12	haemin	15 ft. 1% DEGS 200°	217	450	0.9025	0.44
13	protoporphyrin	5 ft. 1% DEGS 200°	135	305	0.9975	0.44
					Mean =	0.44 ± 0.013

$$r_{IV}:r_V = B_1 f_{IV}:C_1$$
$$= B_1 f_{IV}/C_1:1$$
$$= K:1$$
$$K = r_{IV}/r_V$$

Observations of the relative yields of (IV) and (V) from protoporphyrin, haemin and haematin under various conditions of chromatography, are collected in Table V. Experiments in which (III) was formed have been excluded from consideration because of the difficulty of estimating the total recovery of 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid from varying mixtures of two products. The calculations assume an excess of diazoethane during esterification.

Calculation of the percentage of isomers in a sample of bile pigment

If a sample of bile pigment contains $a\%$ γ isomer and $(100-a)\%$ α isomer and a portion of the esterified oxidation extract gives peak areas B_2 and C_2 mm² at common attenuation and chart speed for (IV) and (V), respectively, then

$$\frac{r_{IV}}{100} \cdot (100-a) : \frac{r_V}{100} \cdot a = B_2 f_{IV} : C_2$$

and

$$(100-a):Ka = C_2:B_2 f_{IV}$$

if f_{IV} is the appropriate response factor.

TABLE VI

THE PERCENTAGE CONTENT OF NON-IXα ISOMERS IN OX GALLSTONE BILIRUBIN ESTIMATED BY GLC ANALYSIS OF ESTERIFIED EXTRACTS OF THE PRODUCTS OF POTASSIUM PERMANGANATE OXIDATION

Sample No.	*GLC conditions*	C_2	B_2	f_{IV}	*percentage content of non-IXα isomer if it is assumed γ*	*percentage content of non-IXα isomer if it is assumed β or γ*
1	15 ft. 1% NGA 200°	10900	96	1.005	1.97	3.94
		9450	80	1.005	2.1	4.2
		6650	56	1.005	1.9	3.8
		17200	127	1.005	1.63	3.26
		13500	80	1.005	1.38	2.76
1	15 ft. 1% DEGS 200°	14700	85	0.9025	1.18	2.36
		27200	169	0.9025	1.25	2.5
		35000	255	0.9025	1.54	3.08
		1900	130	0.9025	1.39	2.78
2	15 ft. 1% DEGS 200°	15900	144	0.9025	1.33	2.66
3	15 ft. 1% DEGS 200°	17000	80	0.98	1.02	2.04
		13900	240	0.98	3.7	5.5
4	15 ft. 1% DEGS 200°	12300	54	0.98	0.97	1.84
5	15 ft. 1% NGA 200°	28000	144	0.9925	1.14	2.28
6	15 ft. 1% NGA 150–200° at 1°/min	3600	20	0.9875	1.18	2.36
		9300	48	0.9875	1.14	2.28
		680	10	0.9875	3.2	6.4
6	15 ft. 1% DEGS 150–200° at 1°/min	3500	43	0.986	2.7	5.4

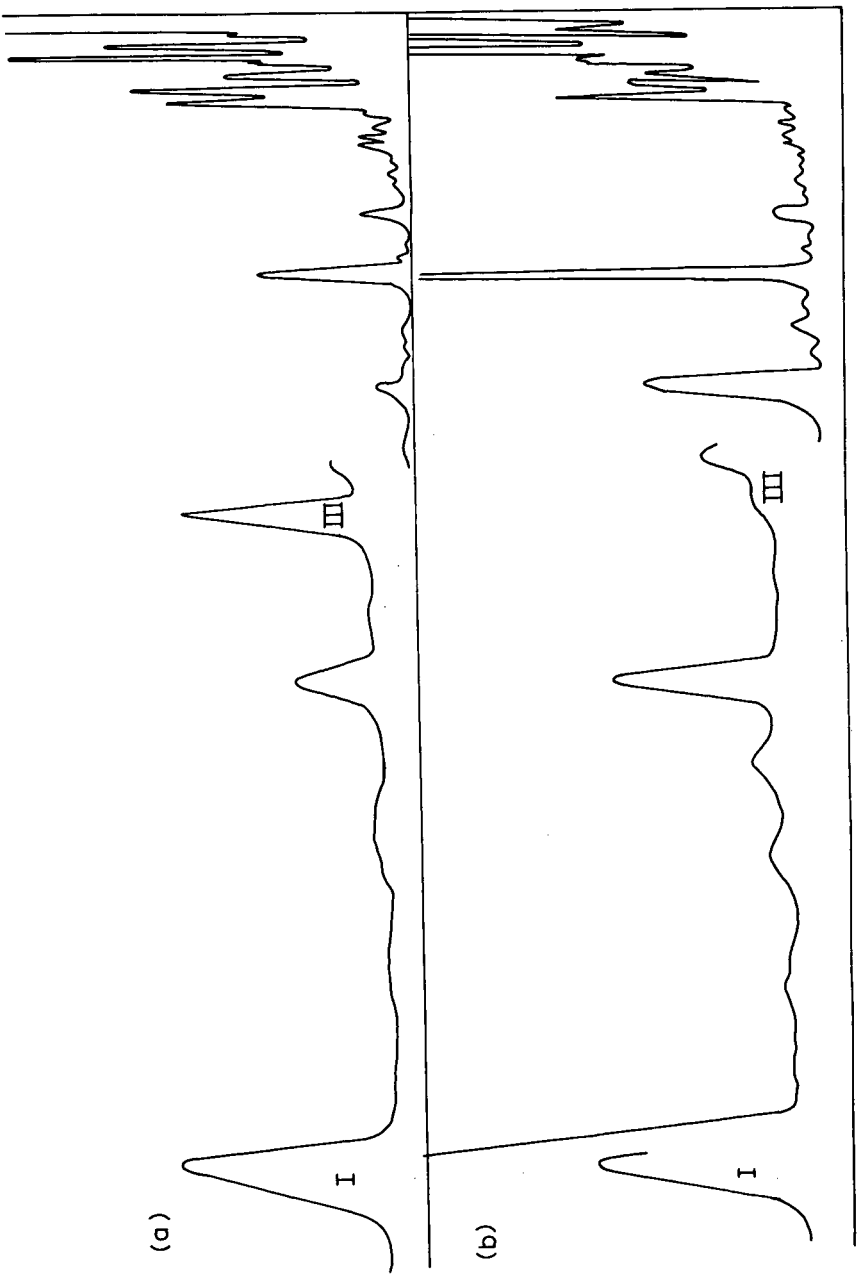

Fig. 2. Chromatograms on 15 ft. 1% DEGS at 200°C, flow rates 15/15/500, chart speed ½ in./min (scale 6 mm = 1 in.). (a) esterified extract of protoporphyrin oxidation product; (b) esterified extract of bilirubin oxidation[4].

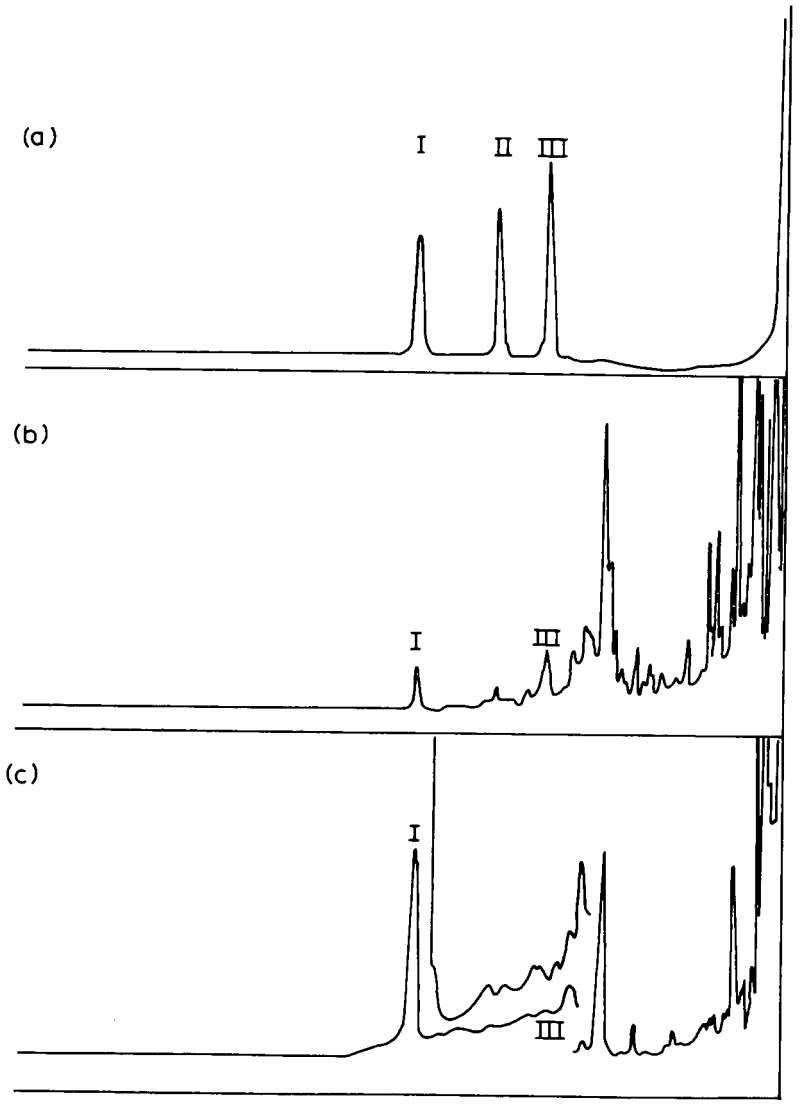

Fig. 3. Chromatograms on 15 ft. 1% NGA, programmed from 150 to 200°C at 1°/min, flow rates 15/15/500, chart speed 12 min/in. (scale 6 mm = 1 in.). (a) esterified pyrrolic acid esters; (b) esterified extract of haematin oxidation[1]; (c) esterified extract of bilirubin oxidation[6].

$$KC_2a = B_2f_{IV}\,(100-a)$$
$$a(KC_2 + B_2f_{IV}) = 100B_2f_{IV}$$

The percentage of γ pigment is therefore given by

$$a = 100B_2f_{IV}/(KC_2 + B_2f_{IV})\%$$

If the sample of bile pigment contains a% β or δ pigment, or if a% of the sample

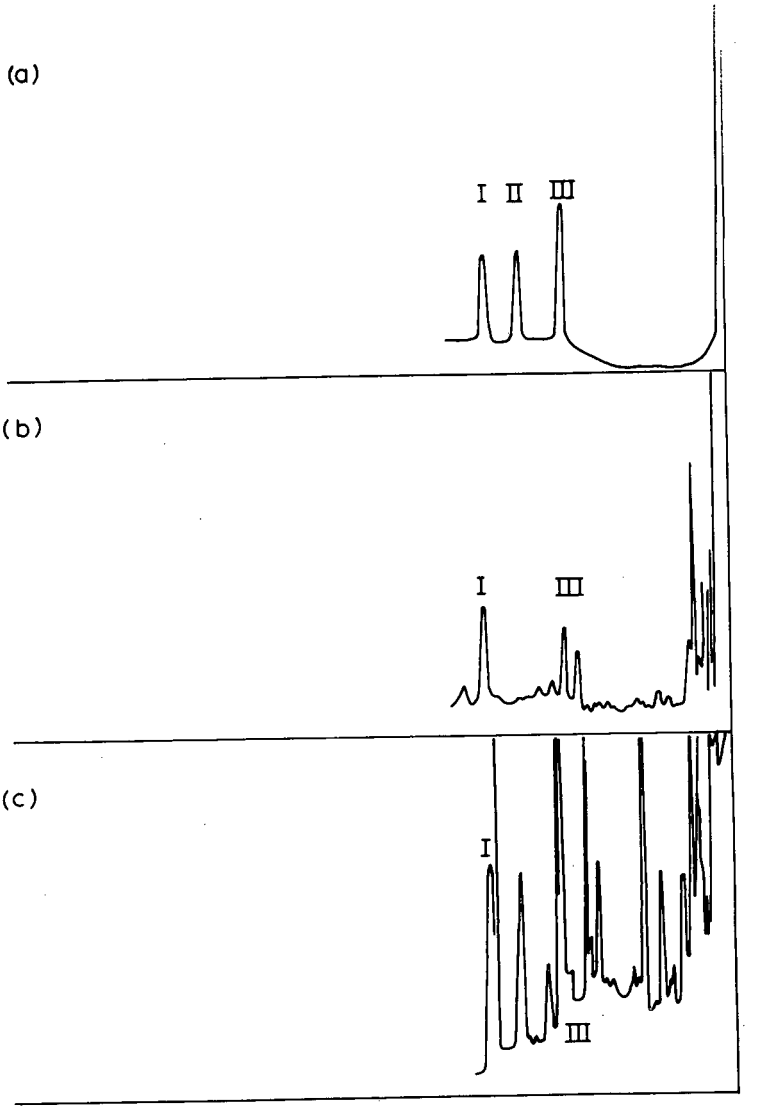

Fig. 4. Chromatograms on 15 ft. 1% DEGS, programmed from 150 to 200°C at 1°/min, flow rates 15/15/500, chart speed 12 min/in. (scale 6 mm = 1 in.). (a) Standard pyrrolic acid esters; (b) esterified extract of haematin oxidation[1]; (c) esterified extract of bilirubin oxidation[6].

consists of a fraction derived from random fission of a porphyrin ring, and $(100-a)\%$ is derived from pure α fission then on oxidation the non-IXα fraction will form $a/2\%$ 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid and $a/2\%$ (V) so that:

$$(100-a+a/2):Ka/2 = C_2:B_2f_{IV}$$

or

$$(100-a/2):Ka/2 = C_2:B_2f_{IV}$$

The percentage of non-IXα pigment will be given by

$$a = 200B_2f_{IV}/(KC_2 + B_2f_{IV})\%$$

The estimation of the percentage content of non-IXα isomers in ox gallstone bilirubin

The results obtained from the attempted measurement of the isomeric content of natural bilirubin are collected in Table VI. 10–100 mg quantities of bilirubin were oxidised, extracted and esterified as described. The residues were dissolved in chloroform 10–100 μl for injection in 1-μl quantities. The peak for (IV) is small in relation to (V) and is poorly resolved from the preceding material as can be seen in Figs. 2, 3 and 4. The peak areas recorded are therefore at best estimated judging the peak base by eye.

Discussion

Small quantities of non-IXα isomer have been detected in natural bilirubin from ox gallstones by GLC analysis of the products of potassium permanganate oxidation. This confirms the original observations of PETRYKA[7]. If the non-IXα fraction is wholly γ isomer it is estimated that it would account for between 1 and 3% of the total bile pigment; if it is a β or δ isomer it would account for between 2 and 6% of the total pigment. Difficulties in measurement of trace peaks and incomplete resolutions render such estimates inaccurate. Moreover, their calculation involves an assumption that cyclic tetrapyrroles afford equal quantities of the initially formed pyrrolic acids in oxidation. The resulting values are the same as those noted by NICHOL AND MORELL[8] in mass spectrometric investigations, but are much higher than the values of 0.1% β isomer and 0.3% δ isomer detected in pig bile bilirubin by O'CARRA AND COLLERAN[9]. These differences may be attributable to species differences but the interpretation of the mass spectrometric analysis of bile pigment isomers has been disputed[9]. NICHOL AND MORELL claim that the product of ascorbate/O_2 oxidation of pyridine haemochrome is a pure β and δ biliverdin whereas RÜDIGER[10] and O'CARRA AND COLLERAN[9] have shown it to be a mixture of all four isomers. These workers have developed techniques for the thin-layer chromatographic separation of all four verdin isomers. The separated isomers can then be further identified by their pyrrole dialdehyde products on dilute sodium dichromate oxidation[10]. In addition O'CARRA AND COLLERAN have been able to distinguish β and δ isomers by comparison with a synthetic β bile pigment.

This adaptation of the NICOLAUS method to GLC, although not achieving greater sensitivity, has made quantitation possible allowing estimation of the relative recovery of the pyrrolic acids. The results indicate that (IV) is about half as stable as (V) to the conditions of oxidation and extraction. Earlier work based on qualitative interpretation of paper chromatograms may, therefore, have led to misleading results.

REFERENCES

1 C. H. GRAY, D. C. NICHOLSON AND R. A. NICOLAUS, *Nature*, 181 (1958) 183.
2 R. A. NICOLAUS, *Rass. Med. Sper.*, 7 (1960) suppl. 2.
3 G. NASHIDA AND R. F. LABBE, *Biochim. Biophys. Acta*, 31 (1959) 519.
4 R. A. NICOLAUS AND R. SCARPATI, *Sugli Acidi Pirrolici IV, Serie 3a, No. 3 degli Atti dell'Academia delle Scienze fisiche ematemitiche di Napoli*, (1962).

5 H. FISCHER AND H. ORTH, *Die Chemie des Pyrrols*, Vol. 1, Akademische Verlagsgesellschaft, Leipzig, 1934, p. 252.
6 J. F. PARCHER AND P. URONE, *J. Gas Chromatogr.*, 2 (1964) 184.
7 Z. J. PETRYKA, *Proc. Soc. Exp. Biol. Med.*, 123 (1966) 464.
8 A. W. NICHOL AND D. B. MORELL, *Biochim. Biophys. Acta*, 184 (1969) 173.
9 P. O'CARRA AND E. COLLERAN, *J. Chromatogr.*, 50 (1970) 458.
10 W. RÜDIGER, in T. W. GOODWIN (Editor), *Porphyrins and Related Compounds*, Academic Press, London, New York, 1968, p. 121.

CHROM. 5384

PREPARATION OF LIPOPHILIC ANION EXCHANGERS FROM CHLOROHYDROXYPROPYLATED SEPHADEX AND CELLULOSE

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SUMMARY

Chlorohydroxypropyl derivatives of Sephadex and cellulose were reacted with ammonia and primary, secondary or tertiary amines to prepare a wide range of lipophilic anion exchangers. These can be used for chromatography in organic solvents, exemplified by the separation of a phospholipid mixture using a dibutylaminohydroxypropyl derivative of Sephadex LH-20.

INTRODUCTION

In a previous communication, the synthesis of chlorohydroxypropyl Sephadex and cellulose from the corresponding hydroxypropyl derivatives was described[1]. It was shown that these lipophilic chlorine-containing derivatives are highly versatile, in that the halogen atom makes further substitution reactions possible. Thus, derivatives with primary, secondary and tertiary amines or quaternary ammonium ions attached to the matrix can be prepared by reacting with ammonia and primary, secondary or tertiary amines. Since this type of reaction represents a new and simple way of preparing a wide range of Sephadex and cellulose ion exchangers suitable for chromatography in organic solvents, it was further investigated.

MATERIALS AND METHODS

Reagents

Hydroxypropyl Sephadex (Sephadex LH-20) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibrous cellulose (Whatman CF 11), amines (purum, Kebo, Stockholm), methanol, chloroform, methylene chloride, ethylene chloride (p.a., Merck), propylene oxide (technical quality, Kebo), epichlorohydrin (purum, Kebo), sodium and potassium hydroxide (p.a., Eka, Sweden) and boron trifluoride ethyl etherate (47% BF_3, Kebo) were used as supplied.

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Solvent regain values

Solvent regain values (g of solvent imbibed in the gel beads per g of dry Sephadex or cellulose derivative) were determined in a stoppered glass tube with sintered glass bottom, essentially as described by HELFFERICH[2].

Elemental analyses

Elemental analyses were kindly carried out by Løvens AS, Ballerup, Denmark.

Preparation of hydroxypropyl cellulose (HP-cellulose)

Fibrous cellulose powder (100 g) was suspended for 1 h in 30% (w/v) aqueous sodium hydroxide, and the excess liquid was then removed by filtration on a Büchner funnel, equipped with a fine-mesh polyethylene filter cloth. The damp cellulose (516 g) was transferred into a 5-l round-bottom glass flask, equipped with a stirrer passing through a bulb condenser. Propylene oxide (2000 ml) and epichlorohydrin (400 ml) were added, and the mixture was refluxed for 12 h with stirring, 800 ml of ethylene chloride being added after the first 2 h. The product (190 g) was collected on a Büchner funnel, washed carefully with water, ethanol and chloroform, and dried at 60°.

Preparation of chlorohydroxypropyl Sephadex LH-20

Sephadex LH-20 (75.7 g) was suspended in methylene chloride (200 ml) in a 1-l round-bottom glass flask, equipped with a stirring rod (a magnetic stirrer will destroy the gel particles) and a separatory funnel. Boron trifluoride ethyl etherate (19 ml) was added after 30 min. 15 min later, a mixture of epichlorohydrin in methylene chloride (35%, v/v; 50 ml) was added slowly via the separatory funnel (1–2 ml/min), and the reaction was allowed to proceed for another 30 min. The product (98.2 g, 8.57% Cl) was collected on a Büchner funnel, washed with ethanol and chloroform and dried at 50°.

Preparation of chlorohydroxypropyl cellulose

This reaction was carried out in a manner similar to that for the preparation of chlorohydroxypropyl Sephadex LH-20. HP-cellulose (10.0 g) was suspended in methylene chloride (25 ml) and reacted with boron trifluoride ethyl etherate (0.6 ml) and epichlorohydrin in methylene chloride (35%, v/v; 34 ml). The product (19.7 g) had a chlorine content of 17.8%.

Preparation of dibutylaminohydroxypropyl Sephadex LH-20

Chlorohydroxypropyl Sephadex LH-20 (13.6 g, 8.57% Cl) was suspended for 30 min in a 15-fold molar excess of dibutylamine (0.51 moles, 86 ml). A solution of potassium hydroxide (2.87 g) in 118 ml of methanol was added to make the final concentration of potassium hydroxide 0.22 *M* and the final volume (in ml) 15 times the weight (in g) of the starting material. After heating to 55° for 3 h with occasional shaking of the reaction vessel, the product was collected on a Büchner funnel and washed with ethanol (1 l), potassium hydroxide (0.1 *M*) in ethanol–water (9:1) (2 l) and ethanol until the eluate was neutral to indicator paper. The product (16.0 g) was dried at 50° for 6 h. Elemental analysis of N was 2.71%. In order to check for absence of chlorine, the product was further washed with 1 *M* aqueous potassium

hydroxide, whereupon the eluate was acidified. No precipitate was obtained upon the addition of silver nitrate. Derivatives using other amines and/or chlorohydroxypropyl cellulose as starting materials were prepared in a similar way.

Column chromatography

10 g of dibutylaminohydroxypropyl Sephadex LH-20 (free base form) were converted to the acetate form by washing on a Büchner funnel with 1000 ml of ethanol–acetic acid (5:1, v/v), followed by ethanol–water (9:1, v/v), until the eluate was neutral to indicator paper, and finally ethanol. The material was dried at 50°. Glass chromatography columns (4–10 mm I.D.), equipped with a porous Teflon or fritted glass end-piece and a solvent reservoir, were packed with a slurry of the Sephadex derivative (acetate form) in chloroform–methanol–water (20:65:35, v/v). Phospholipid samples were applied in a small volume of the eluting solvent. The phospholipid content of each fraction was analyzed by thin-layer chromatography on Silica Gel S using the solvent system chloroform–methanol–acetic acid–water (85:15:10:4.5, v/v). Radioactivity was determined with a gas-flow counter.

RESULTS

Preparation of Sephadex and cellulose ion exchangers

To find the optimal reaction conditions, chlorohydroxypropyl Sephadex LH-20 was reacted with dibutylamine in a number of experiments. Products with a high degree of substitution were obtained if the reactions were carried out in methanol at 50°, using a 15–20-fold molar excess of amine as calculated on the amount of halogen in the chlorohydroxypropyl derivative (Fig. 1A). Maximum substitution was obtained when the reaction mixture was 0.21 *M* with respect to potassium hydroxide (Fig. 1B). Under these conditions, most chlorine groups were replaced by amine within 4 h, after which time no further substitution took place. Several derivatives of Sephadex and cellulose were prepared under these or similar reaction

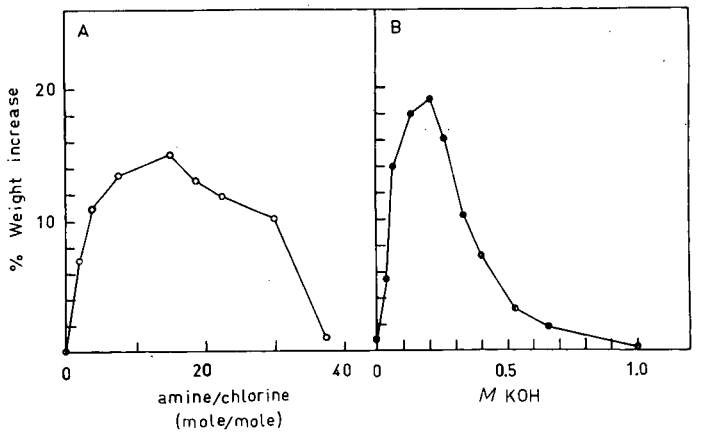

Fig. 1. Effect of concentration of (A) amine (○) or (B) potassium hydroxide (●) on the degree of substitution of chlorohydroxypropyl Sephadex LH-20. The optimal concentration of amine was determined in 0.21 *M* potassium hydroxide. When the influence of base concentration was studied, the Sephadex derivative was reacted with a 15-fold molar excess of dibutylamine.

TABLE I

REACTION OF CHLOROHYDROXYPROPYL SEPHADEX LH-20 WITH DIFFERENT AMINES

Chlorohydroxypropyl Sephadex LH-20 (8.57% Cl = 2.37 mequiv./g) was reacted in methanol with a 15-fold molar excess of amine, the reaction mixture being 0.21 *M* with respect to potassium hydroxide. *b* = free base, *c* = chloride.

Amine	*Weight increase (%)*	*Nitrogen content (%)*	*Capacity (mequiv./g)*[a]	*Solvent regain*									
				Water		*Ethanol*		*Ethylene chloride*		*Benzene*		*Heptane*	
				b	*c*	*b*	*c*	*b*	*c*	*b*	*c*	*b*	*c*
Ammonia	−2	1.57	1.12	0.7	2.0	1.1	1.2	0.9	0.8	0.6	0.4	0.3	0.2
Ethylamine	0.2	2.84	2.03	1.3	3.9	1.5	1.8	1.4	1.1	0.8	0.5	0.2	0.3
Butylamine	8.7	1.95	1.39	0.9		1.5	1.4	1.5	1.0	1.1	0.7	0.2	0.2
Cyclohexylamine	12.6	2.04	1.46	1.1	3.8	1.4	1.7	1.4	1.1	0.8	0.5	0.3	0.3
Ethanolamine	4.7	2.70	1.93	2.5	3.6	1.5	1.5	1.1	0.8	0.9	0.3	0.3	0.2
Aniline	2.0	2.26	1.61										
Diethylamine	5.6	2.27	1.62	1.1	1.8	1.4	1.9	2.1	1.0	0.6	0.5	0.3	0.3
Dibutylamine	15.2	2.71	1.94	0.7	1.5	1.7	1.9	2.4	1.8	1.7	1.2	0.8	0.8
Diethanolamine	14.7	2.24	1.60	1.0	3.3	1.3	1.5	1.0	0.7	0.7	0.3	0.2	0.3
Dimethylcyclohexylamine	5.3	1.27	0.91										
Tributylamine	0.4	0.81	0.58	0.9	0.9	1.5	0.7	1.9	1.2	1.3	0.3	0.4	0.1

[a] As calculated from the nitrogen content.

TABLE II

REACTION OF CHLOROHYDROXYPROPYL CELLULOSE WITH DIFFERENT AMINES

Chlorohydroxypropyl cellulose (18.59% Cl = 5.0 mequiv./g) was reacted in methanol with a 15-fold molar excess of amine, the reaction mixture being 0.21 *M* with respect to potassium hydroxide. b = free base; c = chloride.

Amine	*Weight increase (%)*	*Nitrogen content (%)*	*Capacity (mequiv./g)*[a]	*Solvent regain*									
				Water		*Ethanol*		*Ethylene chloride*		*Benzene*		*Heptane*	
				b	*c*	*b*	*c*	*b*	*c*	*b*	*c*	*b*	*c*
Butylamine	3.9	2.63	1.88	1.4	0.8	0.5	0.4	0.7	0.7	0.8	0.5	0.3	0.3
Cyclohexylamine	8.8	2.49	1.78	1.4	0.7	0.4	0.6	0.6	0.5	0.4	0.3	0.3	0.3
Diethylamine	5.2	2.79	1.50	1.7	0.6	0.7	0.9	0.8	0.6	0.6	0.6	0.4	0.3
Dibutylamine	11.1	1.49	1.01	1.3	0.8	0.4	0.6	0.8	0.8	0.8	0.6	0.5	0.4
Diethanolamine	11.9	2.94	2.10	1.7	0.8	0.5	0.5	1.1	0.9	0.6	0.4	0.3	0.3

[a] As calculated from the nitrogen content.

conditions. The reaction mixtures were worked up quantitatively and the weight increase was noted. The products were further characterized by nitrogen determination, solvent regain measurement and, in some cases, potentiometric titration. The results are summarized in Tables I and II. It is seen that ammonia and primary and secondary amines react readily with the chlorohydroxypropyl derivative, whereas the tertiary amines show low reactivity.

Titrations

The exchange capacities of some dibutylamine derivatives (free base form) were determined by direct potentiometric titration in methanol with 0.1 *M* methanolic HCl. Three derivatives with nitrogen contents corresponding to 2.39, 1.93 and 1.80 mequiv/g had the actual capacities of 2.1, 1.9 and 1.7 mequiv/g, respectively. The results indicate that over 90% of the amine groups are accessible for ion exchange in organic solvents.

Swelling of the products

In this paper, the swelling of the derivatives has been measured as solvent regain, *i.e.* the amount of solvent imbibed in the gel beads per g of dry derivative has been measured after removal of the interstitial fluid by centrifugation. Values below approximately 1.0 (Sephadex derivatives) and 0.6 (cellulose derivatives) indicate poor swelling. The starting material in the synthesis, a halogenated derivative of Sephadex LH-20 or HP-cellulose, is lipophilic and swells in water, alcohols and chlorinated hydrocarbons, but swells poorly in aromatic and aliphatic hydrocarbons. As is seen in the Tables I and II, the ion exchangers swell in the same solvents. In addition, the derivatives prepared from mono- and dibutylamine swell in aromatic hydrocarbons, indicating the importance of the alkyl part of the amine. As expected, the chloride form of the ion exchangers swells best in water[3], whereas the free base form is better swollen in less polar solvents. The same tendency is also seen with the quaternary ammonium derivative prepared from tributylamine, where the

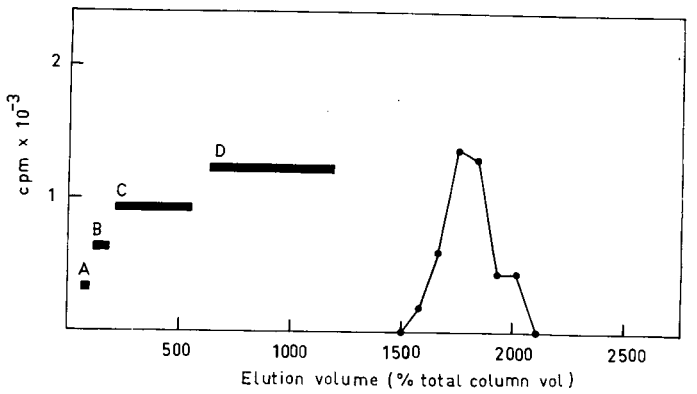

Fig. 2. Separation of egg phospholipids (3 mg) and [4-^{14}C]cholesterol. Heavy bars indicate presence (TLC determination) of: A = lysolecithin, B = lysophosphatidylethanolamine, C = lecithin and D = phosphatidylethanolamine. Column: 225 × 4.4 mm containing 1.0 g of dibutylaminohydroxypropyl Sephadex LH-20 in the acetate form (nitrogen content: 2.71%, w/w). Solvent: Chloroform–methanol–water (20:65:35, v/v). Flow rate: 1.8 ml/h.

hydroxide form (see under "free base", Table I) *e.g.* swells in benzene in contrast to the chloride form.

Applications

In a preliminary experiment, a mixture of egg phospholipids was applied to a column of dibutylaminohydroxypropyl Sephadex LH-20 in its acetate form. The solvent was chloroform–methanol–water (20:65:35, v/v). As is seen in Fig. 2, lysolecithin, lysophosphatidylethanolamine, lecithin, phosphatidylethanolamine and cholesterol are eluted as separate peaks.

DISCUSSION

Synthesis procedure

The chlorohydroxypropyl Sephadex LH-20 starting material contains 8.57% Cl. Although higher chlorine contents can be easily obtained[1], this content was considered suitable, since it corresponds approximately to one chlorine atom per glucose unit. In the subsequent amination procedure, between 50 and 100% of the chlorine atoms are utilized, with the exception of the reaction with tertiary amines, which gives a fairly low yield. The highest degree of substitution is obtained in the reaction with dibutylamine, in which virtually all the chlorine atoms are utilized. Since the optimal reaction conditions were determined using this amine (Fig. 1), somewhat different conditions might be more favourable for the reaction of the chlorohydroxypropyl derivative with other amines. Since every chlorine atom is replaced by an amine group in the dibutylamine reaction, no crosslinking should occur. This is probably true for the other amination reactions too, where the general reaction conditions are identical. The main side reaction is likely to be the hydrolysis of the chlorohydroxypropyl derivative to form the 2,3-dihydroxypropyl derivative.

Applications

Several papers have described the use of cellulose anion exchangers in the separation of phospholipids. However, in solvents of low polarity, such columns are difficult to pack and mechanical problems like channelling frequently disturb the chromatography. Evidently it would be of advantage to use lipophilic ion exchangers[4,5]. The derivatives described in this paper show excellent chromatographic properties in relatively non-polar solvent mixtures. The recovery is good, as determined by chromatography of trace amounts of phospholipids and labelled substances. Further investigations of the use of lipophilic Sephadex and cellulose ion exchangers for the separation of phospholipids and bile acids are currently carried out in this laboratory.

ACKNOWLEDGEMENT

We are much indebted to Professor JAN SJÖVALL for facilities put at our disposal.

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REFERENCES

1 J. ELLINGBOE, B. ALMÉ AND J. SJÖVALL, *Acta Chem. Scand.*, 24 (1970) 463.
2 F. HELFFERICH, *Ionenaustauscher, B.* I, Verlag Chemie, Weinheim/Bergstr., 1959.
3 K. W. PEPPER, H. M. PAISLEY AND M. A. YOUNG, *J. Chem. Soc.*, (1953) 4097.
4 E. NYSTRÖM, *Ark. Kemi*, 29 (1968) 99.
5 J. C. DITTMER, *J. Chromatogr.*, 43 (1969) 512.

CHROM. 5315

AUTOMATED CHROMATOGRAPHY OF AROMATIC ACIDS, ALDEHYDES AND ALCOHOLS WITH AN AMINO ACID ANALYZER

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SUMMARY

Chromatographic separations of a wide range of aromatic compounds (acids, aldehydes, alcohols) have been performed on a spherical cation-exchange resin with a sodium citrate–boric acid buffer, pH 4.53. The organic compounds were automatically determined in the eluate by measuring their light absorption at 280 mμ or their fluorescence at 315 mμ. The method is applicable to the determination of phenolic metabolites from tyrosine, DOPA, catecholamines or tryptophan in biological fluids and tissues.

INTRODUCTION

While organic acids are commonly separated by anion-exchange chromatography[1–9], we have recently demonstrated that acidic and neutral catabolites from dopamine or noradrenalin can be fractionated rapidly by cation-exchange chromatography[10]. This analysis has been performed on a spherical cation-exchange resin, which is used in commercial amino acid analyzers. The catecholamine metabolites were determined automatically in the eluate by measuring their light absorption at 280 mμ. This paper demonstrates that a wide range of aromatic acids, aldehydes and alcohols can be separated in a like manner. Since the new method is very rapid and organic compounds are not destroyed during the column chromatography, it may be of practical importance in the analysis of metabolites from aromatic amino acids or drugs like levodopa.

EXPERIMENTAL

Materials

The sources of materials were as follows: Cation-exchange resin PA-28 (spherical particles of a sulfonated styrene-divinyl-benzene, diameter 16 ± 6 μ) from Beckman Instruments GmbH, Munich, G.F.R.

3,4-Dihydroxyphenylacetic acid; 3-methoxy-4-hydroxyphenylacetic acid; 3,4-dihydroxymandelic acid; 3-methoxy-4-hydroxymandelic acid; 4-hydroxymandelic

acid; kynurenic acid; 5-hydroxyindole-3-acetic acid; caffeic acid; ferulic acid; and 3,4-dimethoxybenzoic acid were from Calbiochem, Los Angeles, Calif., U.S.A. 2,5-Dihydroxyphenylacetic acid; 4-hydroxyphenylacetic acid; 2-methoxymandelic acid; 3-methoxymandelic acid; 3,4-dihydroxyphenylalanine; tyrosine; 5-hydroxytryptophane; xanthurenic acid; 5-hydroxyanthranilic acid; 3,4-dihydroxyphenylglycol; 3-methoxy-4-hydroxyphenylglycol were from Sigma Chemical Company, St. Louis, U.S.A. 3-Hydroxybenzoic acid; 4-hydroxybenzoic acid; 2,6-dihydroxybenzoic acid; 2,4-dihydroxybenzoic acid; 3,5-dihydroxybenzoic acid; 3,4-dihydroxybenzoic acid; and 3-methoxy-4-hydroxybenzoic acid from Fluka AG, Buchs, Switzerland. 3,4-Dimethoxyphenylacetic acid; 4-methoxyphenylacetic acid; 4-methoxymandelic acid were from EGA-Chemie, Heidenheim, G.F.R. 3,4-Dihydroxyphenylethanol and 3-methoxy-4-hydroxyphenylethanol were from A. Kistner AB Fack, Göteborg, Sweden. 3,4-Dihydroxybenzaldehyde; 3-hydroxy-4-methoxybenzaldehyde; 3-methoxy-4-hydroxybenzaldehyde, 2-hydroxybenzoic acid; and 4 methoxybenzyl alcohol were from Dr. Th. Schuchardt AG, Munich, G.F.R. *threo*-3,4-Dibenzoxy-dopaserin, and *erythro*-3,4-dibenzoxy-dopaserin were from Alderich Chemical Company, Milwaukee, U.S.A.

3-O-Methyldopa was kindly supplied by Dr. A. PLETSCHER, Hoffmann-La Roche AG, Basel, Switzerland. 3-Hydroxy-4-methoxybenzoic acid was a gift from Prof. H. THOMAS, the Biochemical Dept. of the University of Ulm, G.F.R.

Chromatography

All separations were performed in water jacketed glass columns (0.9 × 60 cm) of a modified amino acid analyzer of the Unichrom type (Beckman Instruments GmbH, Munich, G.F.R.). A schematic diagram of the chromatographic system is shown in Fig. 1. Columns were filled up to 55 cm with PA 28 resin equilibrated with buffer, pH 3.28. The aromatic compounds at levels of 60 μg to 600 μg, dissolved in 4 ml 0.4 *N* $HClO_4$, were forced into the resin with about 10 atm nitrogen pressure followed in a like manner by two 1 ml washes of buffer, pH 2.2. Then the head space above the resin was filled with buffer, pH 3.28, and elution was carried out as follows: buffer, pH 3.28, for 15 min; Na-citrate–boric acid buffer, pH 4.53, for 100–500 min; and finally with 0.2 *N* NaOH for 60 min. The flow rate was adjusted to 50 ml/h; the

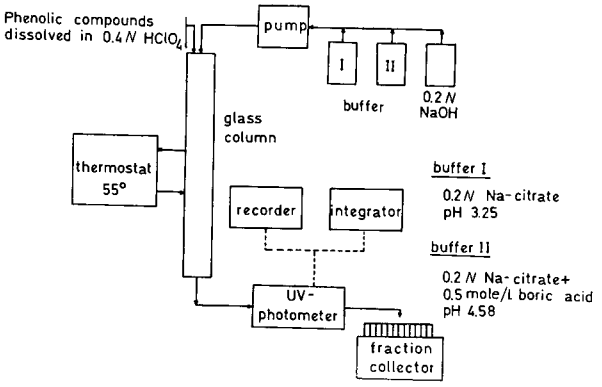


Fig. 1. Schematic diagram of the chromatographic system.

pressure varied between 15 and 20 atm, and the column temperature was 55°. The best separations of aromatic compounds were obtained with 50 mm liquid head space above the resin bed.

Analysis of the column effluent

The eluate from the column was passed through a quartz flow cell (optical path length 10 mm, volume 0.1 ml) of a UV-spectrophotometer (Photometer type DB, Beckman Instruments GmbH, Munich, G.F.R.), and the optical density was measured at 280 mμ. The output of the spectrophotometer was recorded on a laboratory potentiometric recorder and was fed into a digital integrator (Model 125, Beckman Instruments GmbH, Munich, G.F.R.) which automatically prints out the area under each peak. In some experiments the effluent of the flow cell was fractionated by an automatic fraction collector and the fluorescence of each fraction was determined. All fluorometric determinations were performed in an Aminco-Bowman spectrophotofluorometer.

Preparation of buffers

Buffer, pH 2.2. This consists of 19.6 g Na-citrate·$2H_2O$, 5 ml thiodiglycol, 1 g Brij-35, 1 ml *n*-caprylic acid, 850 ml distilled water and 19.6 ml HCl (37%, w/w). The solution was titrated with 6 *N* HCl to pH 2.2 and made up to 1 l.

Na-citrate buffer, pH 3.28. 392.2 g Na-citrate·$2H_2O$, 100 ml thiodiglycol, 20 g Brij-35, 2 ml *n*-caprylic acid, 18 l distilled water and 246.5 ml HCl (37%, w/w) were all mixed together and the solution was adjusted to pH 3.28 and made up to 20 l.

Na-citrate–boric acid buffer, pH 4.53. This is the same as for buffer, pH 3.28, but with the addition of 167.5 ml HCl (37%, w/w) and 600 g boric acid and adjustment to pH 4.53.

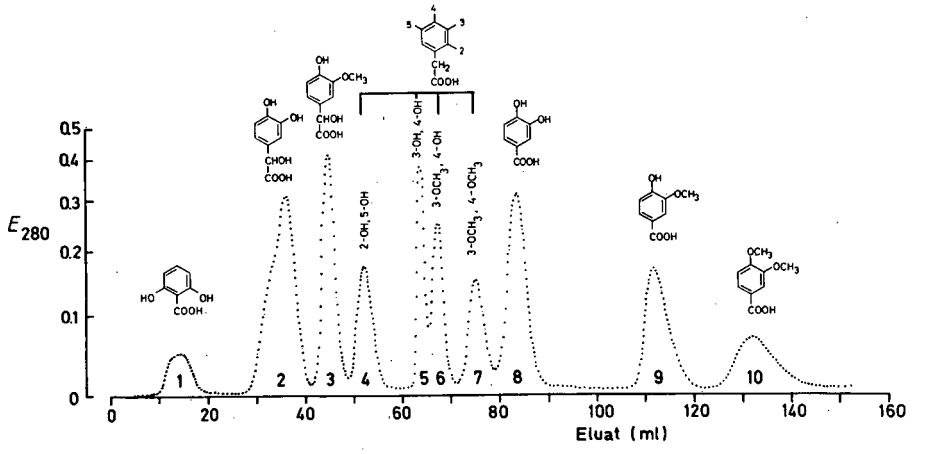


Fig. 2. Separation of ten phenolic acids by cation exchange chromatography. Conditions: column, 0.9 × 60 cm; resin, 16 ± 6 μ PA-28; eluent, 0–15 min Na citrate buffer, pH 3.28, 15–150 min Na-citrate–boric acid buffer, pH 4.53; flow rate, 50 ml/h; pressure 15–20 atm; column temperature, 55°. 1 = 2,6-Dihydroxybenzoic acid (200 μg); 2 = 3,4-dihydroxymandelic acid (600 μg); 3 = 3-methoxy-4-hydroxymandelic acid (400 μg); 4 = homogentisic acid (200 μg); 5 = homoprotocatechuic acid (200 μg); 6 = homovanillic acid (200 μg); 7 = homoveratric acid (200 μg); 8 = protocatechuic acid (200 μg); 9 = vanillic acid (200 μg); 10 = veratric acid (200 μg).

All water used for buffer formulation was deionized on a mixed bed resin and distilled in a quartz double distillation apparatus.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram obtained in the analysis of the mixture of ten phenolic acids. This mixture, containing 200–600 μg of each acid, was separated in about 3 h. 2,6-Dihydroxybenzoic acid, a rather strong acid (dissociation constant 5.0×10^{-2}), was eluted first, then the mandelic acids—3,4-dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid. Phenolic derivatives of phenylacetic acid were in general eluted ahead of the corresponding benzoic acids. Even related compounds, from the chemical point of view, like 2,5-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid or vanillic acid and isovanillic acid (11 and 12 in Fig. 5), are well separated. A typical example of the fractionation of phenol alcohols and phenol aldehydes is shown in Fig. 3. Aldehydes are retained for longer by the resin than alcohols.

The elution times of fifty aromatic compounds are reported in Table I. It

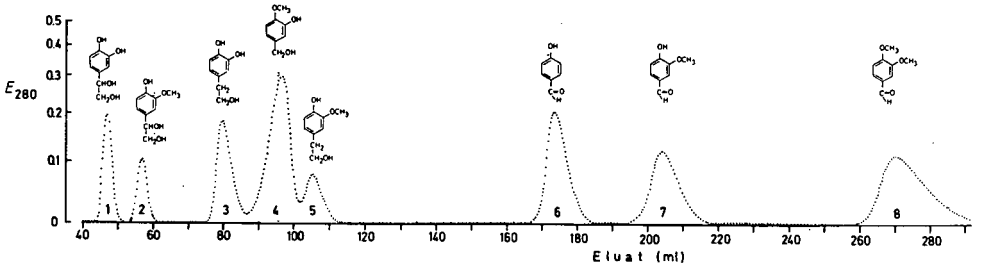


Fig. 3. Separation of five phenolic alcohols and three phenolic aldehydes by cation exchange chromatography. For conditions see Fig. 2. 1 = 3,4-Dihydroxyphenylglycol (200 μg); 2 = 3-methoxy-4-hydroxyphenylglycol (200 μg); 3 = 3.4-dihydroxyphenylethanol (200 μg); 4 = 3-hydroxy-4-methoxybenzyl alcohol (100 μg); 5 = 3-methoxy-4-hydroxyphenylethanol (200 μg); 6 = 4-hydroxybenzaldehyde (60 μg); 7 = vanillin (100 μg); 8 = veratraldehyde (200 μg).

would seem that more compounds may be separated by our chromatographic system than listed in this table, since Table I contains only those substances related to our biochemical problems and detectable by UV absorption. It can be seen from Table I that some relationship between the chemical structure of a compound and its elution time can be demonstrated. The different retention times of the various hydroxybenzoic acids are good examples of the influence of the position of the phenolic hydroxyl group on the elution pattern. In monohydroxybenzoic acids retention time increases in the sequence 2-OH < 3-OH < 4-OH. This order is still preserved in dihydroxybenzoic acids and in methoxy-hydroxybenzoic acids. In accordance with this, the retention time of 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) is significantly shorter than that of 4-hydroxy-3-methoxybenzoic acid (vanillic acid).

Dissociation constants taken from a handbook[11] are indicated in Table I for some compounds, since we presumed a relationship between the dissociation and retention on the PA-28 column. A vague relationship of this kind can be seen in the group of the hydroxybenzoic acids. As a rule weak acids are retained by the column

AB IE I

ELUTION TIME OF VARIOUS AROMATIC COMPOUNDS

For chromatographic conditions see Fig. 2.

Compound	*Elution time (min)*	*Compound*	*Elution time (min)*	K_{diss}[a]
Hydroxyphenylacetic acids		Hydroxybenzoic acids		
2,5-Dihydroxy	70	2,6-Dihydroxy	20	5.0×10^{-2}
4-Hydroxy	78	2-Hydroxy	73	2.0×10^{-3}
3,4-Dihydroxy	80	2,4-Dihydroxy	74	5.2×10^{-4}
3-Methoxy-4-hydroxy	85	3,5-Dihydroxy	88	9.1×10^{-5}
3,4-Dimethoxy	93	3-Hydroxy	91	4.4×10^{-5}
4-Methoxy	98	3,4-Dihydroxy	106	3.3×10^{-5}
		4-Hydroxy	142	1.1×10^{-4}
Hydroxymandelic acids		3-Hydroxy-4-methoxy	138	3.2×10^{-5}
3,4-Dihydroxy	40	3-Methoxy-4-hydroxy	150	3.0×10^{-5}
3-Methoxy-4-hydroxy	60	3,4-Dimethoxy	160	3.6×10^{-5}
4-Hydroxy	62			
2-Methoxy	63	Hydroxybenzaldehydes		
3-Methoxy	68	3,4-Dihydroxy	115	2.8×10^{-8}
4-Methoxy	72	4-Hydroxy	214	2.2×10^{-9}
		3-Hydroxy-4-methoxy	245	
Amino acids		3-Methoxy-4-hydroxy	258	4.0×10^{-8}
threo-Dopaserine	68			
erythro-Dopaserine	72	Phenol alcohols		
DOPA	90	3,4-Dihydroxyphenylglycol	53	
Tyrosine	100	3-Methoxy-4-hydroxyphenyl glycol	66	
3-O-Methyldopa	117	3,4-Dihydroxyphenylethanol	93	
5-Hydroxytryptophane	198	3-Methoxy-4-hydroxyphenyl-ethanol	126	
Tryptophane	285	3-Hydroxy-4-methoxybenzyl-alcohol	101	
Other aromatic acids		4-methoxybenzylalcohol	140	
4-Hydroxyphenyllactic acid	67			
Xanthurenic acid	72			
Kynurenic acid	136			
5-Hydroxyindole-3-acetic acid	146			
5-Hydroxyanthranilic acid	158			
4-Hydroxyphenylpyruvic acid	209			
Caffeic acid	320			
Urocanic acid	346			
Ferulic acid	417			

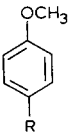
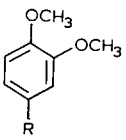
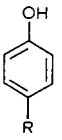
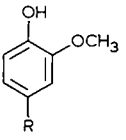
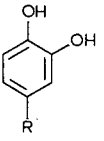
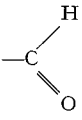
[a] Dissociation constants in aqueous solution at 20° or 25°. All values are taken from ref. 11.

for longer than stronger ones. But a comparison of aldehydes with the benzoic acids in Table I immediately demonstrates that dissociation cannot be of great importance for this kind of separation. The dissociation constants of aldehydes are much smaller than those of acids, for example, protocatechaldehyde has a K_{diss} of 2.8×10^{-8} and protocatechuic acid has a K_{diss} of 3.3×10^{-5}, while their retention times shown in Table I are rather similar, 115 min for protocatechaldehyde and 106 min for protocatechuic acid.

From Table I it is evident that those compounds which have particularly short retention times have several OH-groups in the benzene ring and several OH- or COOH-groups in the side chain. Table II is another illustration of this fact. In this table 3,4-dihydroxymandelic acid, which has two phenolic OH-groups in the benzene

TABLE II

INFLUENCE OF THE NUMBER OF HYDROXYL OR CARBOXYL GROUPS PER MOLECULE ON ITS RETENTION TIME ON A PA-28 COLUMN

OH or COOH groups in the side chain	*R*	*Aromatic OH-groups*				
		0		*1*		*2*
						
0		—	333	214	245	115
1	—COOH		160	142	146	106
	—CH_2—CH(—NH_2)—COOH	—	—	100	117	90
	—CH_2—CH_2OH	—	—	—	126	93
	—CH_2—COOH	98	93	78	85	80
	—CH(OH)—CH(NH_2)—COOH	—	—	—	—	68 (*threo-*) 72 (*erythro-*)
	—CH(OH)—CH_2OH	—	—		66	53
2	—CH(OH)—COOH	72	—	62	60	40
	—CH_2—CH(OH)—COOH	—	—	67	—	—

ring and one carboxylic group and one OH-group in the side chain, has the shortest retention time (40 min). 3,4-Dimethoxybenzaldehyde, which contains neither free phenolic OH-groups nor OH- or COOH-groups in the side chain, has the longest elution time. Thus, in general our chromatographic system seems to separate aromatic compounds according to their polarity.

The content of boric acid in the buffer is not critical, since many compounds listed in Table I can be separated by the Na citrate buffer, pH 4.53, even without boric acid.

Many substances, the separation of which is described in this paper, are rather unstable and they may be destroyed during the chromatographic separation. Therefore the percentage recovery was measured for the following compounds[10]: 3,4-dihydroxymandelic acid, 92%; 3-methoxy-4-hydroxymandelic acid, 100%; 3,4-dihydroxyphenylglycol, 83%; 3-methoxy-4-hydroxyphenylglycol, 97%; 3,4-dihydroxy-

phenylacetic acid, 90%; 3-methoxy-4-hydroxyphenylacetic acid, 100%; 3,4-dihydroxyphenylethanol, 100%; and 3-methoxy-4-hydroxyphenylethanol, 88%. Thus, from a practical point of view this chromatographic system has a recovery of around 100%.

Between 60 and 600 μg of each compound were separated in the chromatograms shown in Figs. 2 and 3. This rather high quantity is necessary as long as a UV flow cell is used as a detector. Much smaller quantities of each compound are sufficient, if their fluorescence is measured in the eluate. Aromatic compounds which contain mobile (π) electrons are normally fluorescent. Fluorescence of benzene derivatives depends on the freedom of the π-electrons. Hydroxylic groups or methoxylic groups enhance benzene fluorescence, carboxylic groups diminish it[12]. An example of the detection of aromatic compounds in the eluate by fluorescence measurements is given in Fig. 4. In this case 10–30 μg of each compound was applied on the column and the fluorescence of 1.5 ml fractions of the eluate was measured in a spectrofluorometer.

The chromatographic system described in this paper was initially developed to separate acidic and neutral metabolites of catecholamines. Fig. 5 gives a typical example of this kind of application. It demonstrates that the catecholamine precursor DOPA can easily be separated from all its neutral and acidic biological metabolites.

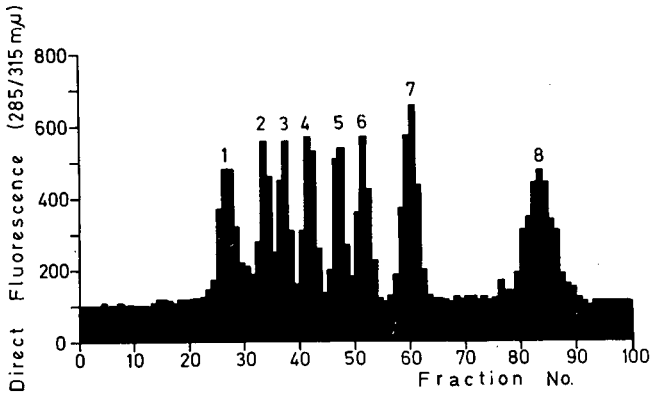


Fig. 4. Determination of aromatic compounds in PA-28 column eluate by measurement of the fluorescence at 315 mμ. Activation at 280 mμ: for chromatographic conditions see Fig. 2. 1 = 3,4-Dihydroxymandelic acid (30 μg); 2 = 3,4-dihydroxyphenylglycol (30 μg); 3 = 3-methoxy-4-hydroxymandelic acid (10 μg); 4 = 3-methoxy-4-hydroxyphenylglycol (10 μg); 5 = 3,4-dihydroxyphenylacetic acid (20 μg); 6 = 3-methoxy-4-hydroxyphenylacetic acid (10 μg); 7 = 3,4-dihydroxyphenylethanol (30 μg); 8 = 3-methoxy-4-hydroxyphenylethanol (10 μg).

This separation may even be of some practical interest, since BIRKMAYER AND HORNYKIEWICZ[13] as well as BARBEAU *et al.*[14] and more recently COTZIAS *et al.*[15] have demonstrated that Parkinson's disease can efficiently be treated with DOPA. Thus, DOPA metabolism in mammals was reinvestigated by several groups[16,17]. All these investigations suffered from the lack of efficient analytical methods for the separation of neutral and acidic metabolites from DOPA.

From a practical point of view another advantage of our separation procedure

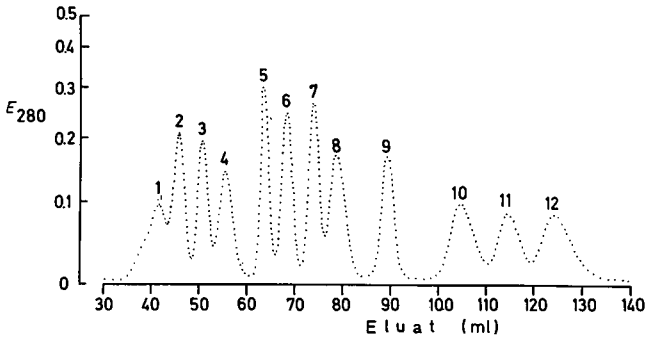


Fig. 5. Separation of twelve acidic and neutral metabolites from DOPA. For conditions see Fig. 2. 1 = 3,4-dihydroxymandelic acid (200 μg); 2 = 3,4-dihydroxyphenylglycol (200 μg); 3 = 3-methoxy-4-hydroxymandelic acid (200 μg); 4 = 3-methoxy-4-hydroxyphenylglycol (400 μg); 5 = 3,4-dihydroxyphenylacetic acid (200 μg); 6 = 3-methoxy-4-hydroxyphenylacetic acid (200 μg); 7 = 3,4-dihydroxyphenylethanol (200 μg); 8 = DOPA (200 μg); 9 = 3-O-methyldopa (200 μg); 10 = 3-methoxy-4-hydroxyphenylethanol (200 μg); 11 = isovanillic acid (400 μg); 12 = vanillic acid (100 μg).

is worth mentioning. In experiments on catecholamine metabolism metabolites are generally extracted from tissues by 0.4 N $HClO_4$. These perchloric acid extracts can be analyzed immediately without further treatment with our system.

ACKNOWLEDGEMENTS

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REFERENCES

1 J. INCZÉDY, *Analytische Anwendungen von Ionenaustauschern*, Verlag der Ungarischen Akademie der Wissenschaften, Budapest, 1964.
2 N. KIRSHNER AND McC. GOODALL, *J. Biol. Chem.*, 226 (1957) 905.
3 McC. GOODALL AND H. ALTON, *Biochem. Pharmacol.*, 17 (1968) 905.
4 McC. GOODALL, W. R. HARLAN AND H. ALTON, *Circulation*, 38 (1968) 592.
5 McC. GOODALL AND H. ALTON, *J. Clin. Invest.*, 92 (1969) 1761.
6 H. THOMAS, *J. Chromatogr.*, 34 (1968) 106.
7 O. SAMUELSON AND L. THEDE, *J. Chromatogr.*, 30 (1967) 556.
8 B. CARLSSON AND O. SAMUELSON, *Anal. Chim. Acta*, 49 (1970) 247.
9 E. MARTINSSON AND O. SAMUELSON, *Chromatographia*, 3 (1970) 405.
10 H.-W. LANGE, H. F. K. MÄNNL AND K. HEMPEL, *Anal. Biochem.*, 38 (1970) 98.
11 J. D'ANS AND E. LAX (Editors), *Taschenbuch für Chemiker und Physiker*, Springer, Berlin, Heidelberg, New York, 1967.
12 S. UDENFRIEND, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, London, 1962.
13 W. BIRKMAYER AND O. HORNYKIEWICZ, *Wien. Klin. Wochenschr.*, 73 (1961) 787.
14 A. BARBEAU, T. L. SOURKES AND G. F. MURPHY, in J. DE AJURIAGUERRA (Editor), *Les catecholamines dans la maladie de Parkinson. Monamines et système nerveux centrale*, Masson, Paris, 1962, p. 247.
15 G. C. COTZIAS, M. H. VAN WOERT AND L. M. SCHIFFER, *New Engl. J. Med.*, 276 (1967) 374.
16 G. BARTHOLINI AND A. PLETSCHER, *J. Pharm. Pharmacol.*, 2 (1969) 324.
17 R. J. WURTMAN, C. CHOU AND C. ROSE, *J. Pharmacol. Exp. Therap.*, 174 (1970) 351.

CHROM. 5318

AN IMPROVED METHOD FOR THE AUTOMATED ANALYSIS OF SUGARS BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A method for the separation and determination of several mono-, di- and trisaccharides is described. Step-wise elution systems with borate buffers at high temperatures have been developed for the ion-exchange chromatography of sugars, utilizing a Dowex 1 X4 resin. Total analysis time was 250, 336 and 610 min for the respective systems developed.

Analysis of the column effluent was performed with a Technicon AutoAnalyzer using the orcinol colorimetric method. It has been established that a linear relationship exists between peak area or net peak height and different varying amounts of sugars.

INTRODUCTION

At present, there are several chromatographic methods available that utilize ion-exchange resins for the quantitative analysis of sugars[1–9]. ARWIDI AND SAMUELSON[1] have been able to obtain a separation of monosaccharides by means of partition chromatography using strong anion- or cation-exchange resins. In spite of good resolution of several monosaccharides over a reasonable time-period, this chromatographic system presents considerable technical difficulties: high temperature with volatile solvents, high pressure in the column, low solubility of oligosaccharides in the water–ethanol mixture used as eluent and rapid wearing out of the manifold tubes due to the high concentration of ethanol in the eluate.

For this reason, preference has been accorded to various methods of sugar separation on the borate form of anion-exchange resins. After the pioneering work of KHYM AND ZILL[2], many authors developed chromatographic techniques with strong anion-exchange resins in the borate form for the separation and quantitative determination of a mixture of sugar–borate complexes[3–9]. The development of these chromatographic methods now permits a separation of complex mixtures of mono-, di- and trisaccharides. Most of the methods described by these authors are based on gradient elution systems.

KHYM *et al.*[3] and OHMS *et al.*[4] used a concentration gradient of borate buffers, whereas KESLER[5] and LEE *et al.*[6] described a system in which the sugars were eluted

with a pH-concentration gradient of borate buffers. SYAMANANDA *et al.*[7] carried out the chromatographic separation with a chloride-ion gradient in borate buffers at constant pH and concentration.

Separation and quantitative determination of neutral saccharides has also been achieved by step-wise elution with borate buffers, whether[8] or not[9] these contained chloride ions.

The main drawback of these chromatographic procedures was their slowness. Either the chromatography itself required a great deal of time or the column had to be regenerated after each run.

This paper deals with an improved method for automated quantitative analysis of sugars using an ion-exchange resin in the borate form. A borate buffer of fixed borate concentration or a step-wise elution system is used here for the resolution of several mono-, di- and trisaccharides.

EXPERIMENTAL

Apparatus

This work was performed with a Technicon AutoAnalyzer. The analytical train consisted of the following Technicon modules: (a) positive micropump; (b) chromatographic column; (c) proportioning pump; (d) adjustable heating bath equipped with a 1.7 mm × 12 m reaction coil; (e) colorimeter with filter at 420 mμ and 15 mm light path cuvet; (f) one-channel recorder. The chromatographic system and analytical manifold are schematically represented in Fig. 1.

The transport lines were made from glass tubes, where possible; when glass tubes could not be used, the lines consisted of acidflex transmission tubes. Chromatographic analyses were performed with 0.6 × 110 cm or 0.6 × 140 cm borosilicate

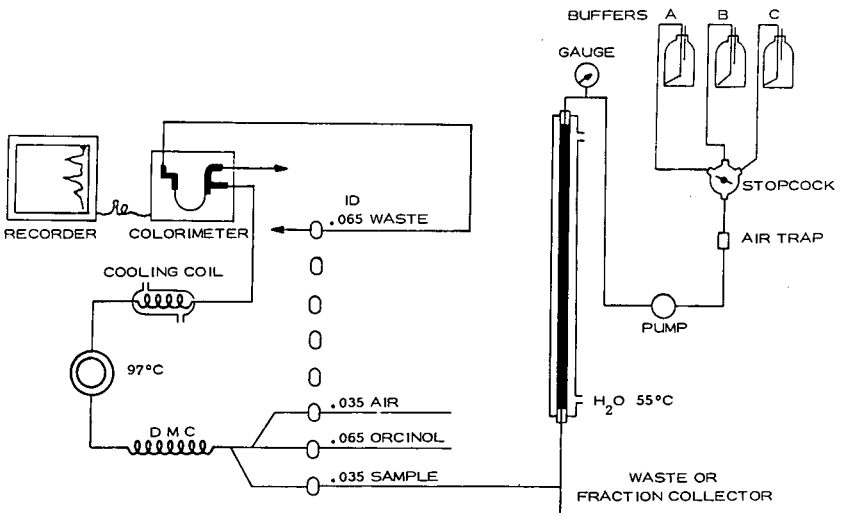

Fig. 1. Diagram of the chromatographic and analytical systems.

glass columns. The columns were glass jacketed; the water circulating within the jacket at 55° came from a superthermostat, Bühler type A5.

Resins

The resin used for the chromatographic analysis described in this paper was Dowex 1 X4 (200–400 mesh: Dow Chemical Co.). Other resins such as AG 1 X8 and AG 1 X4 (minus 400 mesh: Bio-Rad Laboratories); Dowex 1 X2 and Dowex 2 X8 (200–400 mesh: Dow Chemical Co.) and De-Acidite FF (3–5 cross-linked, minus 200 mesh: Permutit Co. Ltd.) were also tested.

Chemicals and reagents

Sugars were supplied by Nutritional Biochemicals Corp.; all were of 96% purity minimum. The other chemical products used were purchased from B. D. H. Laboratories. The colour reagent for the autoanalyzer consisted of 1 g of orcinol per litre of sulphuric acid 70% (v/v); this solution was prefiltered in a G4 glass filter and stored in a brown glass bottle.

Buffers

Buffer A: 0.11 *M* potassium tetraborate–0.17 *M* boric acid, pH 8.80; buffer B: 0.0255 *M* potassium tetraborate–0.125 *M* boric acid, pH is adjusted to 8.40 with 1 *M* KOH; buffer C: 0.053 *M* potassium tetraborate–0.088 *M* boric acid, pH 8.80.

Preparation of ion-exchange resins and columns

A large batch of resin was fractionated by repeated settling in aqueous ethanol (50%) to obtain a narrower distribution of uniform beads; the finest particles were isolated and used for chromatography. The purified resin (about 200 ml of settled beads) was treated on a büchner funnel with 2 *N* NaOH until no further chloride ions were detectable in the filtrate (with silver nitrate–nitric acid); after washing with deionized water to remove excess NaOH, the resin was converted to the borate form by washing with 0.5 *M* boric acid until the pH reached 4.2–4.3 and it then was equilibrated with a suitable buffer (A or C).

The resin, de-aerated under vacuum at 55°, was poured into the column and packed in 3–5 sections. During the packing of the columns, buffer A or C was pumped through the resin at a flow rate of 45 ml/h. The final height of the packed resin bed was 100–102 cm for the shorter column and 130–132 cm for the longer one. Final equilibration of the column was carried out with 200 ml of the selected buffer (A or C).

Chromatographic conditions

Sugar mixtures of known quantities were dissolved in 0.05 *M* potassium tetraborate solution and adjusted to pH 7.2–7.3 with boric acid; appropriate volumes of this solution (0.05 – 1.5 ml) were used for chromatography.

In this work, three chromatographic systems were used.

System 1: One-buffer system. The column was equilibrated with buffer A; after absorption of the sample under nitrogen pressure and washing with buffer A, elution was started with the same buffer at 60 ml/h. We were able to obtain a good separation of two disaccharides and seven monosaccharides in 250 min.

System 2: Two-buffer step-wise elution system. The sample was poured on the

TABLE I

ELUTION TIMES OF SUGARS (min)

Sugar	*System 1*	*System 2*	*System 3*
Sucrose	55	63	96
Trehalose	—	—	107
Melezitose	—	77	125
Raffinose	—	95	149
Maltose	74	118	206
Rhamnose	—	—	220
Lactose	—	130	240
Stachiose	—	—	270
Ribose	87	155	291
Mannose	115	190	338
Fructose	136	211	380
Arabinose	151	222	391
Galactose	169	241	412
Xylose	191	267	438
Glucose	230	306	484
Gentiobiose	—	—	505
Melibiose	—	—	585

column equilibrated with buffer A; elution was started with buffer B at 45 ml/h. After 90 min, elution was continued with buffer A at 60 ml/h. These operating conditions allowed a good chromatographic resolution of twelve sugars in 336 min. Regeneration of the column was not required.

System 3: Three-buffer step-wise elution system. In this case, chromatographic conditions were as follows: buffer C was used for equilibration of the column (the complete regeneration of the resin after each run required about 180 min); elution was started with buffer B at 60 ml/h; the buffer was changed after 225 min to buffer C, increasing the flow rate to 75 ml/h, and after 260 min to buffer A with the same flow rate. This separation was carried out with the longer column.

All separations were carried out at a column temperature of 55°.

RESULTS

The elution time of the sugars investigated in this paper was established by adding two components at a time and at different concentrations, to a synthetic mixture of sucrose, mannose and glucose, the elution time of which had previously been determined. The elution times of the seventeen sugars, examined in the three systems, are given in Table I. The values are given as the number of minutes required to elute the peak of each sugar on the chromatogram.

The resolution possibilities that can be obtained with chromatographic system 1 are shown by the chromatogram in Fig. 2. A typical chromatogram of a mixture of two trisaccharides, three disaccharides and seven monosaccharides obtained under the elution conditions of system 2 is shown in Fig. 3. In addition to these, it has been established that, under the same experimental conditions, trehalose presents an elution volume similar to sucrose; stachiose is eluted with ribose, and rhamnose is only partially separated from lactose. The chromatographic behaviour of seventeen sugars can be seen in Fig. 4. The separation of the complex mixture components was possible by using the chromatographic conditions of system 3.

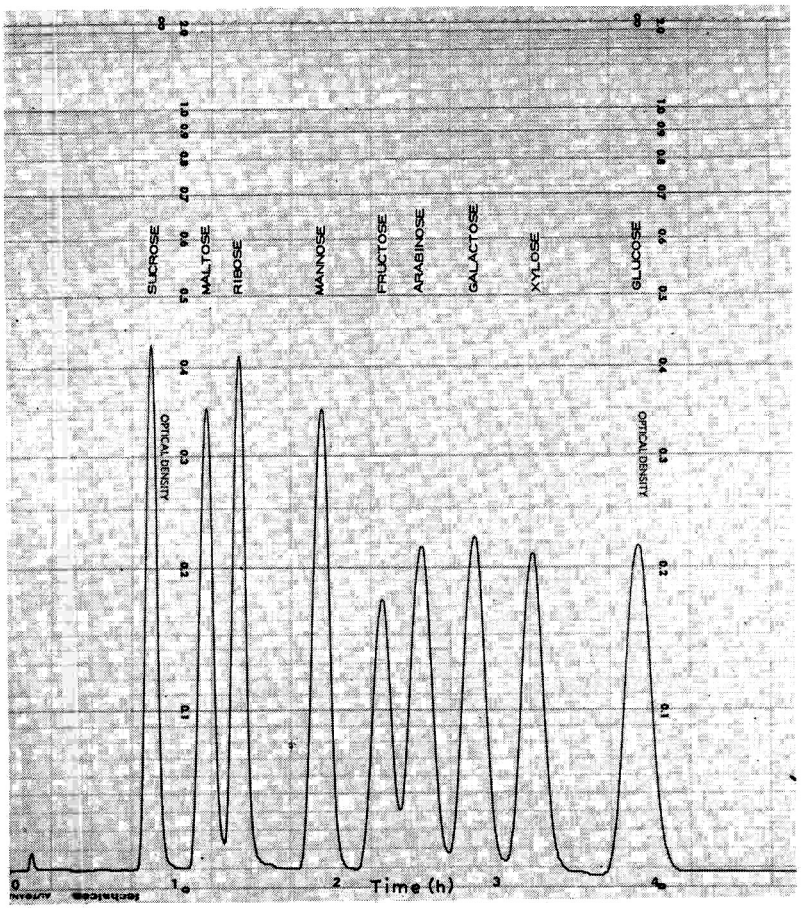

Fig. 2. Chromatogram of a nine-component synthetic mixture (system 1). Pentoses: 20 μg; other sugars: 50 μg.

Quantitative estimation

In the quantitative evaluation of the chromatograms, the areas of the chromatographic peaks (optical density *vs.* time) were usually integrated by multiplying the height (net optical density) of the peak by the width (mm) at half of the nett height[10].

The ratio of the integrated values relative to different quantities of sugars was determined by a series of five chromatograms of a nine-component standard solution performed under the conditions of system 1. Quantities of each sugar added to the column ranged from 5 to 50 μg.

The relationship between peak area and the respective amounts of sugars is shown in Fig. 5. It must be emphasized that since each peak width at half height is constant and independent of the amount of sugar, the net peak height is proportional to the concentration: thus a quantitative calibration of the chromatograms can also be obtained from the nett absorbance measured from base line to peak-tip (Fig. 6).

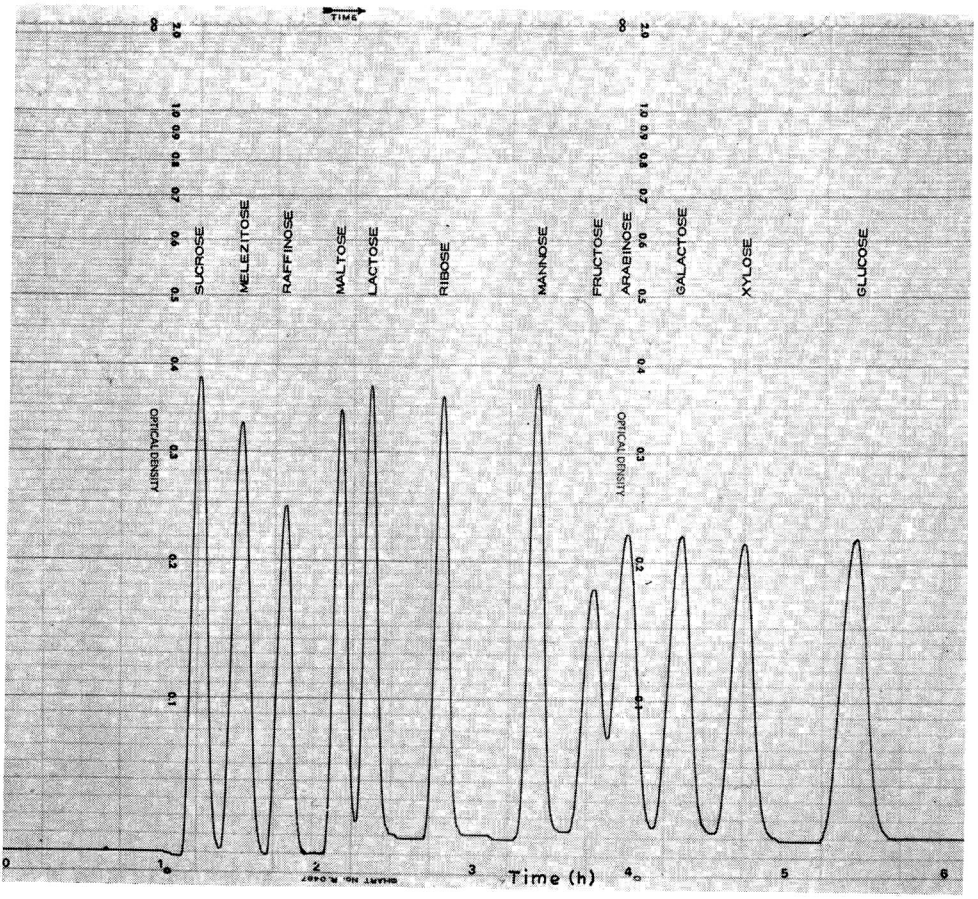

Fig. 3. Chromatography of a mixture of twelve sugars (system 2). Concentrations as in Fig. 2.

A linear relationship between sugar amount and optical density exists in the range investigated of 5–50 μg.

Reproducibility of the results

Reproducibility of the results with known mixtures of sugars was checked from chromatograms similar to that reported in Fig. 2. The reproducibility test was carried out by six runs made with the same amount of each sugar; the results are summarized in Table II. The values refer to the calculation of the mean areas and peak heights corresponding to 20 μg of pentoses and 50 μg of all the other sugars present in the mixture.

The pH change of the eluate during a chromatographic analysis performed with systems 2 and 3 is recorded in Fig. 7.

DISCUSSION

The ion-exchange chromatographic systems reported in this paper can be

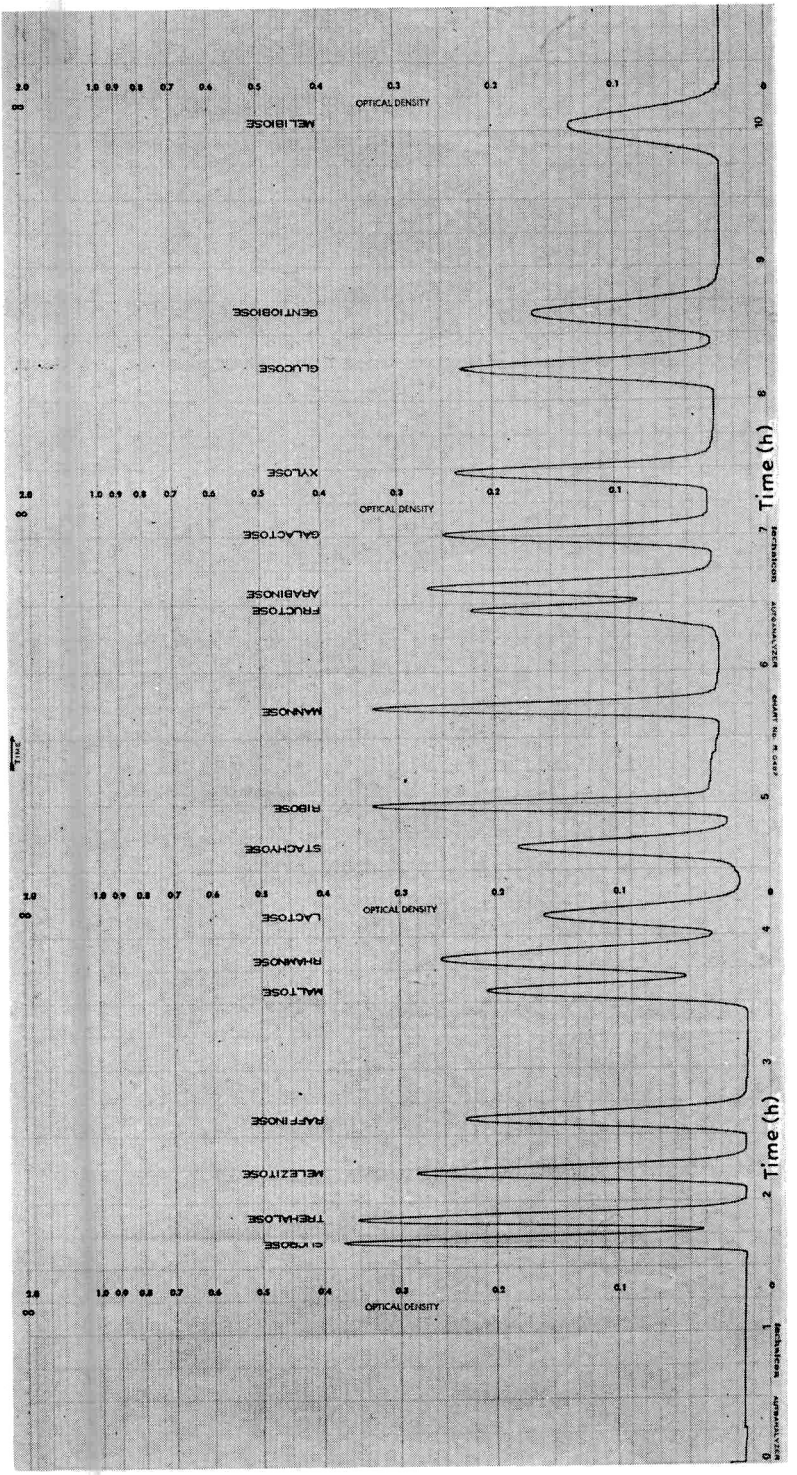

Fig. 4. Chromatographic separation (system 3) of a seventeen-component mixture. Concentration of each sugar as in Fig. 2.

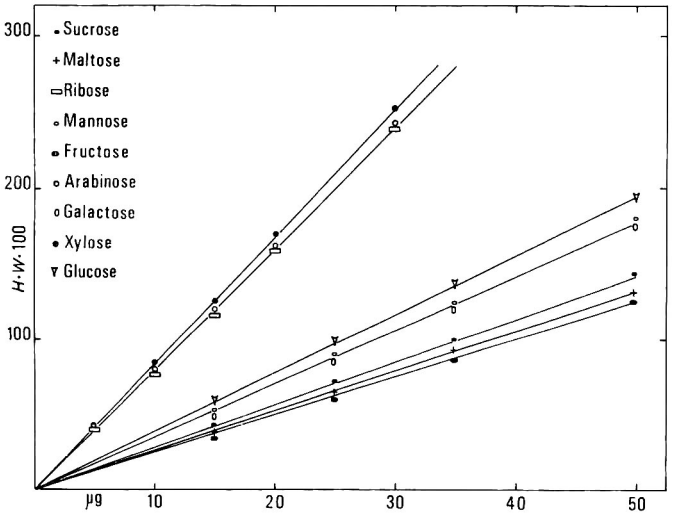

Fig. 6. Relationship between amount of sugar and peak height.

employed successfully for the complete separation of complex mixtures of sugars.

Elution system 1 does not provide a good resolution of di- and trisaccharides, but it can be used for the quantitative analysis of natural monosaccharides or monosaccharides released by chemical or enzymatic hydrolysis of polysaccharides or glycoproteins. System 2 is a versatile tool for the separation of multicomponent sugar mixtures in less than 6 h. Resolution, particularly for di- and trisaccharides, is very high. Moreover, under these conditions, the column need not be regenerated and a series of analyses can be carried out continuously.

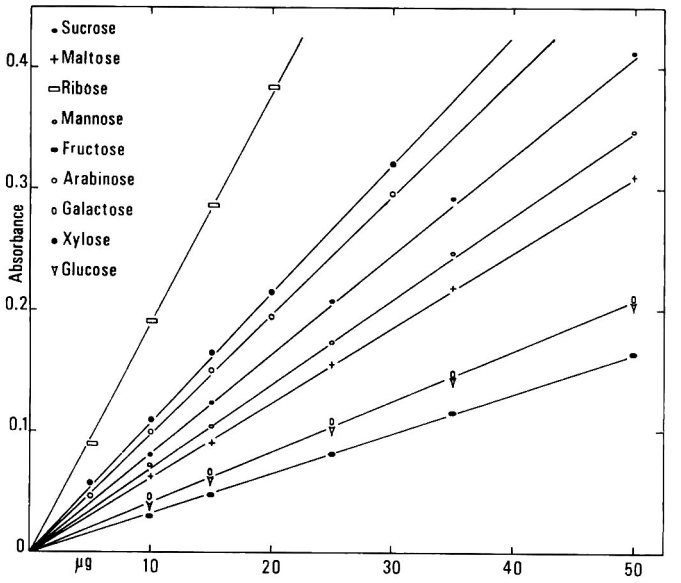

Fig. 6. Relationship between amount of sugar and peak height.

TABLE II

REPRODUCIBILITY OF THE AREAS AND HEIGHTS OF THE ELUTION PEAKS

Sugar	*Mean areas* $H \cdot W \cdot 100$	*S.D.%*	*Mean peak heights absorbance*	*S.D.%*
Sucrose	136	0.7	0.405	2.4
Maltose	128	3.1	0.319	1.8
Ribose	150	2.0	0.390	2.0
Mannose	172	1.1	0.351	3.8
Fructose	97	3.8	0.161	3.2
Arabinose	131	3.0	0.190	2.7
Galactose	143	3.3	0.204	2.8
Xylose	145	1.3	0.208	2.9
Glucose	168	0.5	0.209	2.9

Analysis of more complex mixtures of sugars requires the three-buffer step-wise system. We were able to obtain the complete separation of seventeen sugars, in about 600 min with a 0.6 × 140 cm column. After each run the column had to be re-equilibrated with buffer C. It must be emphasized that the elution volumes of trehalose, rhamnose and stachiose were greatly dependent on the pH changes occurring in the column whenever the ionic strength of the buffer changed (Fig. 7).

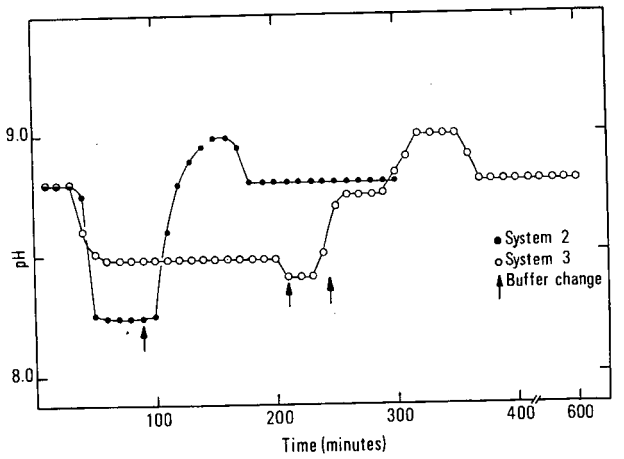

Fig. 7. pH changes of column effluent in step-wise chromatographic systems.

It was observed that, if the pH value is lower than 8.4, trehalose overlaps sucrose, and rhamnose overlaps lactose. Similarly stachiose was eluted with ribose at pH values higher than 8.8. In the step-wise elution systems, a slight shift of the chromatogram baseline was constantly observed.

The resolution power of the resin remained constant even after several months; nevertheless the column had to be repacked after 70–80 analyses, since after a while lactose undergoes transformation to a new compound which was eluted between

lactose and ribose. This drawback is not remedied by periodic replacement of the top centimetre of the blackened resin bed.

The other resins investigated presented low or very low separation power. Under the chromatographic conditions described here, AG 1 X8, Dowex 2 X8 and Amberlite CG 400 did not demonstrate any valid chromatographic resolution whatsoever. Moderate resolution was shown in a progressive order by Permutit, AG 1 X4 and Dowex 1 X2.

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REFERENCES

1 B. ARWIDI AND O. SAMUELSON, *Sv. Papperstidn.*, 68 (1965) 330.
2 J. X. KHYM AND L. P. ZILL, *J. Amer. Chem. Soc.*, 74 (1952) 2090.
3 J. X. KHYM, R. L. JOLLEY AND C. D. SCOTT, *Cereal Sci. Today*, 15 (1970) 44.
4 J. I. OHMS, J. ZEC, J. V. BENSON AND J. A. PATTERSON, *Anal. Biochem.*, 20 (1967) 51.
5 R. B. KESLER, *Anal. Chem.*, 39 (1967) 1416.
6 Y. C. LEE, J. F. MCKELVY AND D. LANG, *Anal. Biochem.*, 27 (1969) 567.
7 R. SYAMANANDA, R. C. STAPLES AND R. J. BLOCK, *Contrib. Boyce Thompson Inst.*, 21 (1962) 363.
8 R. B. KESLER, *Automation in Analytical Chemistry, Technicon Symposia 1965*, Mediad Inc., New York, 1965, p. 174.
9 E. F. WALBORG, D. B. RAY AND L. E. ÖHRBERG, *Anal. Biochem.*, 37 (1970) 320.
10 S. MOORE, D. M. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1190.

CHROM. 5376

SÉPARATION DES POLYSACCHARIDES ET ACIDES NUCLÉIQUES PAR CHROMATOGRAPHIE SUR HYDROXYAPATITE

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SUMMARY

Separation of polysaccharides and nucleic acids by chromatography on hydroxyapatite

Partial separation of polysaccharides and nucleic acids can be obtained by column chromatography on hydroxyapatite at room temperature. However, the separation depends on the phosphate concentration of these substances, their degree of denaturation, and the presence or absence of phospholipids in bacterial extracts.

INTRODUCTION

On sait que l'affinité des acides nucléiques pour l'hydroxyapatite (HTP) est liée à une plus ou moins grande concentration des ions PO_4^{2-} dans leur molécule. La séparation des différents acides nucléiques se fait par augmentation des ions PO_4^{2-} dans les tampons d'élution, et donc compétition au niveau de l'HTP avec les ions PO_4^{2-} des acides nucléiques[1]. En postulant que les polysaccharides de paroi des germes Gram négatif sont moins chargés en phosphates que les acides nucléiques, on peut parvenir à une séparation effective des polysaccharides et des acides nucléiques.

L'expérimentation entreprise, dont nous donnons ici la méthode d'exploration et les résultats obtenus, montre que ce postulat n'est pas vrai pour tous les polysaccharides et acides nucléiques et que cette méthode de séparation semble être fonction de la concentration en phosphates de ces substances.

MATÉRIAUX ET MÉTHODES

Ce travail a été réalisé sur des phases acqueuses d'extraction selon la classique méthode de WESTPHAL ET JANN[2], à partir d'*Escherichia coli* et de *Bordetella pertussis*.

Chromatographies

L'hydroxyapatite utilisée est le produit commercial Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Calif.).

Les colonnes sont de 15.5 à 16.5 cm × 2 cm, équilibrées en tampon phosphate

de potassium 0.005 *M*, pH = 6.8. Les différents tampons d'élution sont préparés à partir de solutions de phosphates mono- et di-potassique, aux concentrations molaires désirées et mélangées pour obtenir le même pH pour tous les tampons.

Les chromatographies sont effectuées comparativement à 4° et 21°, car on sait que la température de chromatographie joue un rôle important dans la séparation des substances sur HTP[3]. Des fractions de 5 ml sont recueillies à l'aide d'un collecteur de fractions.

Méthodes analytiques

Dosage des sucres. On utilise la technique à l'anthrone selon MOKRASCH[4]. L'intensité de coloration est mesurée au spectrophotomètre à 620 mμ, comparativement à une gamme-étalon de solution de glucose à des concentrations allant de 40 à 200 μg.

Dosage des acides ribonucléiques (ARN). C'est la technique décrite par KAMALI ET MANHOURI[5] qui est utilisée. Le ribose est dosé par une réaction à l'orcinol comparativement à une gamme-étalon d'ARN, l'intensité colorimétrique se mesurant au spectrophotomètre à 670 mμ.

Dosage des acides desoxyribonucléiques (ADN). Les ADN sont dosés par la technique à la diphenylamine et acide perchlorique selon BURTON[6] avec mesures colorimétriques à 600 mμ.

Dosage des protéines. La technique D'ITZHAKI ET GILL[7] qui est utilisée est une micro-méthode au biuret avec mesure photométrique à 310 mμ.

Mesures spectrophotométriques. Elles sont réalisées, aussi bien dans l'ultra-violet que dans le visible, avec un spectrophotomètre Beckman DB-G. La mesure des densités optiques des différentes chromatographies est effectuée à 260 mμ.

Concentration des produits de chromatographie

Lorsque ce n'est pas spécifiquement signalé, les pics d'élution sont concentrés par pervaporation à 4° et dialyse contre eau distillée pour éliminer les phosphates. En effet, des essais de concentration sur Dia-Flo (Amicon Corp., Lexington, Mass.) avec membrane PM 10 montrent que les ARN aussi bien que les sucres traversent cette membrane et ceci dans des proportions importantes: 57% pour les ARN et 60% pour les sucres.

RÉSULTATS

Chromatographie d'extrait de B. pertussis

Nous avons réalisé une chromatographie à température ambiante (21°) d'un extrait polysaccharidique de *B. pertussis*. Les dosages de sucres et d'ARN effectués sur la totalité des paliers d'élution, montrent que l'on a séparation effective d'une partie des acides nucléiques et des sucres, puisque 47% de ces derniers sont élués en tête de chromatographie dans le palier 0.005 *M*, contaminés seulement par 6% d'ARN. Cependant, plus de la moitié des polysaccharides (51%) est éluée en même temps que la majeure partie des ARN (63%) avec le palier 0.2 *M* (Tableau I).

Chromatographies d'extrait d'E. coli

Les deux chromatographies effectuées comparativement à 4° et 21°, ne mon-

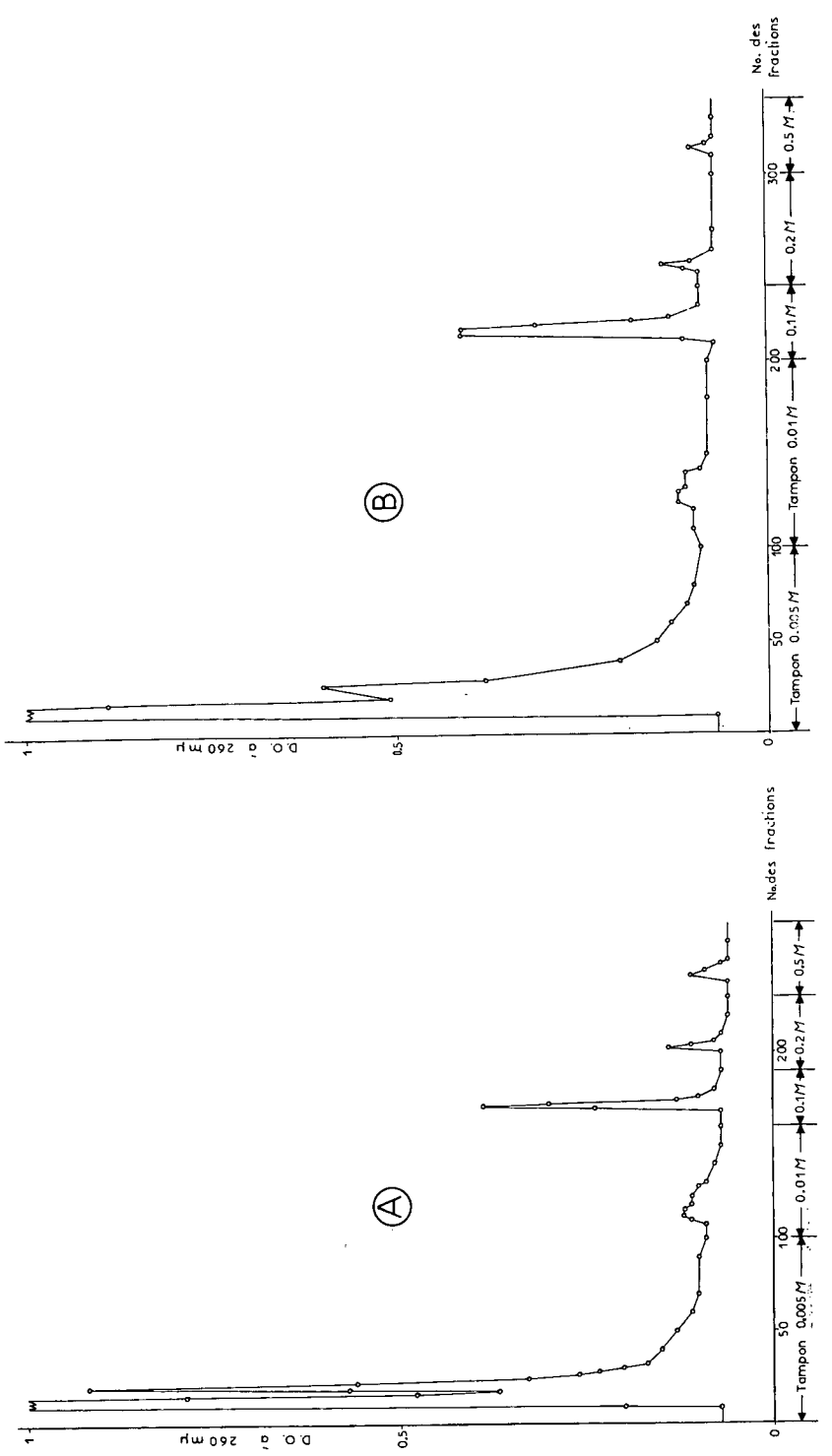

Fig. 1. Chromatographies d'extraits d'*E. coli*. Colonnes de 16 × 2 cm. Vitesse d'élution : 25 ml/h. Volume des fractions : 5 ml. Volumes mis en chromatographie : 10 ml, contenant ARN (6.1 mg); ADN (1.8 mg); polysaccharides (1 mg); protéines (○). (A) = chromatographie à 4°; (B) = chromatographie à 21°.

TABLEAU I

CHROMATOGRAPHIE D'EXTRAIT DE *B. Pertussis*

Dosage des sucres et des ARN dans les différentes fractions.

Matériel	*A.R.N.*		*Sucres*	
	µg totaux	%	*µg totaux*	%
Extrait mis sur colonne	7063	100	726	100
Palier 0.005 *M*	457.65	6.3	346.1	47.6
Palier 0.01 *M*	10.1	0.1	0	—
Palier 0.1 *M*	365.55	5.1	0	—
Palier 0.2 *M*	4456.8	63	374.4	51.5
Palier 0.5 *M*	161.24	2.2	131.8	18
Total	5451.74	76.7	852.3	117.1

trent pas de différence dans leur courbe d'adsorption à 260 mμ (Fig. 1, A et B). Cependant, les dosages de sucres, d'ARN et d'ADN effectués sur les fractions des différents paliers, montrent que ces substances sont généralement plus fortement adsorbées sur l'HTP à 4° qu'à 21°. On obtient ainsi une meilleure séparation des différentes substances à l'élution, et plus spécialement des polysaccharides, en opérant les chromatographies à température ambiante: en effet, 45% environ des polysaccharides sont élués dans le palier 0.005 *M*, contaminés seulement par 8% d'ARN, mais 31% d'ADN. De plus, à 21°, les rendements obtenus à la chromatographie sont meilleurs qu'à 4°: en effet, pour les sucres, on obtient 108.8% de récupération à 21°, contre 65.5% à 4°. Les rendements pour l'ARN restent pauvres et

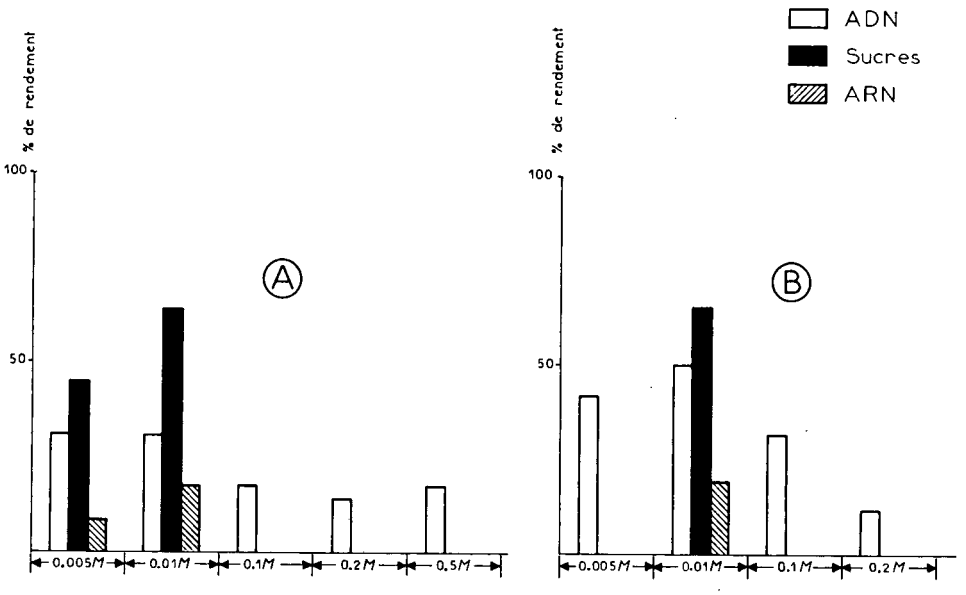

Fig. 2. Pourcentages de rendement des chromatographies d'extraits d'*E. coli*. (A) = chromatographie à 21°; (B) = chromatographie à 4°.

sont faiblement augmentés par l'élévation en température de la chromatographie: 25.9% à 21°, contre 19.5% à 4°. Seuls les ADN présentent une récupération totale, quelle que soit la température: 111.8% à 21° et 135.6% à 4° (Fig. 2A et B).

DISCUSSION

Les travaux de BERNARDI[1,8,9], concernant la chromatographie des acides nucléiques sur hydroxyapatite, ont montré que la séparation obtenue est fondée sur l'interaction des groupements phosphates négatifs des acides nucléiques, avec la charge positive à la surface des cristaux d'hydroxyapatite, sans aucune intervention directe ni des bases, ni des sucres. Ce même auteur[10], a montré d'autre part, que l'adsorption des polypeptides et des protéines sur hydroxyapatite était fonction de la charge en groupements aspartate et glutamate des protéines: plus une protéine contient de groupements carboxyliques et plus elle est fortement adsorbée à la surface des cristaux d'hydroxyapatite.

Les acides nucléiques, dans la composition desquels entre en moyenne 10% de phosphore[11], sont généralement élués de l'hydroxyapatite à 0.1–0.2 *M* phosphates. Occasionnellement, quelques fractions mineures d'ADN natif sont éluées à 0.5 *M*[8].

On pouvait donc penser qu'en partant d'extraits bactériens dépourvus de protéines, tels que ceux que nous avons utilisés, on pourrait obtenir une séparation des acides nucléiques et des polysaccharides, ces derniers, *a priori* moins chargés en phosphore, étant élués avant les acides nucléiques.

Cette hypothèse a été partiellement vérifiée pour ce qui concerne les extraits de *B. pertussis*, puisque 47.6% des polysaccharides ont été élués dans le palier 0.005 *M* (Tableau I). Ces polysaccharides correspondent certainement à des substances faiblement chargées en phosphore, pour lesquels l'hypothèse de produits de dégradation des polysaccharides natifs doit aussi être envisagée. Les ARN élués entre 0.005 *M* et 0.1 *M* représentent des fragments de polynucléotides plus ou moins dégradés selon le palier d'élution, tandis que la majeure partie des ARN (63%) est éluée à 0.2 *M* et représente des ARN natifs. Cependant, près de 70% des polysaccharides sont encore élués dans les paliers 0.2–0.5 *M*. Ils correspondent à des substances fortement chargées en phosphore. On peut penser que ces substances fortement phosphorylées (plus de 10% en phosphates, si l'on établit la correspondance avec les acides nucléiques) ne sont pas composées uniquement de polysaccharides, mais de lipopolysaccharides, dont la fraction phospholipidique vient s'ajouter à la charge en phosphore.

Il en va de même pour les extraits d'*E. coli*, passés en chromatographie à 21°. En effet, à cette température, la totalité des polysaccharides (108%) est éluée entre les paliers 0.005 et 0.10 *M*, contaminés par 26% d'ARN et 62% d'ADN. Ces résultats sont comparables avec ceux obtenus avec les extraits de *B. pertussis*. Dans le cas d'*E. coli*, les ARN et ADN élués avant 0.1–0.2 *M* phosphates, correspondent aussi certainement à des fragments de polynucléotides.

La comparaison des chromatographies d'extraits phénoliques d'*E. coli* aux différentes températures, montre que: (a) l'adsorption des polysaccharides est généralement plus forte à 4° qu'à 21°, puisque 40% environ des sucres ne sont pas élués à 0.5 *M* phosphates. Il paraît donc préférable d'effectuer les chromatographies à température ambiante. (b) Les ARN sont peu élués de la colonne (19.5% de récu-

pération à 4°, 26% environ à 21°). Et ce qui est élué correspond à des fragments d'ARN. On peut donc en conclure que le reste des ARN, probablement sous une forme dégradée, s'est combiné avec des substances fortement phosphatées, telles que des phospho-lipides. (c) La température de chromatographie n'affecte pas l'élution des ADN dans leur ensemble. On remarquera seulement que plus de 60% de ces ADN sont décomposés en polynucléotides plus ou moins polymérisés, puisqu'ils sont élués dans les fractions 0.005–0.01 *M* phosphates.

Ces séparations sur hydroxyapatite mettent donc en évidence une dénaturation importante des substances contenues dans les extraits phénoliques, puisque selon BERNARDI[1], l'hydroxyapatite ne provoque pas de cassures dans les longues molécules d'ARN. Cette dégradation est variable selon les germes utilisés, puisque l'on recueille 63% d'ARN natif dans les extraits de *B. pertussis* et rien à partir des extraits d'*E. coli*. D'autre part, cette dégradation des acides nucléiques et des sucres dans les extraits phénoliques est confirmée par le fait que lors d'une concentration de l'extrait brut sur Dia-Flo, avec membrane PM 10, on retrouve près de 60% de sucres et de polyribonucléotides dans le filtrat. Il paraît peu probable que la méthode d'extraction soit en cause dans ces dénaturations d'acides nucléiques; une dégradation par les opérations de congélation–décongélation au stockage paraît plus plausible.

CONCLUSION

La chromatographie sur hydroxyapatite d'extraits bactériens de germes Gram négatif permet effectivement une séparation des polysaccharides et des acides nucléiques, dans la mesure où ces derniers ne sont pas trop dégradés par les opérations de purification et de conservation qui précèdent. Toutefois, cette séparation s'avère incomplète. On peut penser que cela est dû à la présence de phospholipides, qui modifient la charge en phosphates des polysaccharides et des polynucléotides. Cette méthode de séparation n'est donc pas applicable avec le même bonheur à tous les germes. Il semble que le rendement de purification soit essentiellement fonction de l'intégrité moléculaire des polysaccharides et acides nucléiques en présence; et du taux de concentration en phosphates des polysaccharides ou des lipopolysaccharides, qui, pour une séparation maximale, doit être inférieur à celui des acides nucléiques. D'autres expériences sont en cours dans notre laboratoire pour vérifier cette hypothèse.

REMERCIEMENTS

Nous remercions Monsieur le Docteur G. AYME, qui nous a fourni les extraits bactériens utilisés dans ce travail, ainsi que Mademoiselle N. BROCARD pour son excellente collaboration technique.

RÉSUMÉ

La chromatographie sur colonne d'hydroxyapatite à température ambiante permet une séparation partielle des polysaccharides et acides nucléiques: mais, cette

séparation est fonction de la concentration en phosphates de ces substances, de leur degré de dénaturation et de la présence ou absence de phospholipides dans l'extrait bactérien traité.

BIBLIOGRAPHIE

1 G. BERNARDI, *Biochim. Biophys. Acta*, 174 (1969) 449.
2 O. WESTPHAL ET K. JANN, in R. L. WHISTLER (Editor), *Methods in Carbohydrate Chemistry*, Vol. 5, Academic Press, New York, 1965, p. 83.
3 G. VIDAL, *J. Chromatogr.*, 41 (1969) 279.
4 L. C. MOKRASCH, *J. Biol. Chem.*, 208 (1954) 55.
5 M. KAMALI ET H. MANHOURI, *Clin. Chem.*, 15 (1969) 390.
6 K. BURTON, *Biochem. J.*, 62 (1956) 315.
7 R. F. ITZHAKI ET D. M. GILL, *Anal. Biochem.*, 9 (1964) 401.
8 G. BERNARDI, *Biochim. Biophys. Acta*, 174 (1969) 423.
9 G. BERNARDI, *Biochim. Biophys. Acta*, 174 (1969) 435.
10 G. BERNARDI ET T. KAWASAKI, *Biochim. Biophys. Acta*, 160 (1968) 301.
11 H. R. MAHLER ET E. H. CORDES, *Biological Chemistry*, Harper & Row, New York, 1969, p. 124.

CHROM. 5317

GEL FILTRATION OF OLIGOSACCHARIDES ON CROSS-LINKED STARCH AND CELLULOSE

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SUMMARY

Cross-linking of microcrystalline cellulose and starch with epichlorohydrin under alkaline conditions afforded gels suitable for gel permeation chromatography of oligosaccharides. Successful separation of a mixture of mono-, di-, and trisaccharides using water as a mobile phase showed that starch modified in the described manner is an excellent gel permeation material. Although the chromatographic properties of the cellulose gel under investigation were comparable with those of other cellulose gels used for gel permeation chromatography, a complete separation of a monosaccharide from a trisaccharide could not be obtained.

INTRODUCTION

Ever since PORATH AND FLODIN[1,2] carried out, for the first time, gel filtration on polysaccharides using dextran cross-linked with epichlorohydrin, this technique has been widely used as an important means of fractionation of various substances.

Several types of cross-linked dextran, with different degrees of swelling, both in water and organic solvents are on the market under the trade name Sephadex.

DETERMAN *et al.*[3] tried to use cellulose gel for gel permeation chromatography (GPC). He precipitated cellulose from a Schweizer's solution with benzoic and acetic acid and obtained a solid, chemically stable gel in the form of spherical particles, diameter 0.03–0.3 mm. However, this gel with a pore diameter, in water, of some hundreds of nanometers, is only suitable for GPC of substances with a molecular weight in the range 10^4–10^7.

MARTIN AND ROWLAND[4] observed that the cross-linking of cellulose with formaldehyde decreases the permeability of large particles and, at the same time, improves the separation of low molecular weight substances. They cross-linked decrystallized cellulose with formaldehyde and obtained a gel suitable for GPC of oligosaccharides of molecular weight up to 10^3.

Oligosaccharides in the molecular weight range of 10^2–10^3 were separated on starch gel for the first time by LATHE AND RUTHVEN[5] who used a column packing of maize starch.

The most advantageous way in which cellulose and starch may be modified in order to acquire gel permeation properties is obviously cross-linking. Epichlorohydrin proves to be a suitable cross-linking agent.

The purpose of the present work is to compare the gel permeation properties of cross-linked microcrystalline cellulose, cross-linked starch and Sephadex G 15 with one another.

EXPERIMENTAL

Materials

Two supports used in this work, C_E (cellulose gel) and S_E (starch gel) have been prepared by cross-linking microcrystalline cellulose and potato starch, respectively.

C_E: the cellulose gel. The cellulose gel was made from microcrystalline cellulose according to ref. 6. Microcrystalline cellulose was made alkaline in an inert solvent and treated, under stirring, with epichlorohydrin. The molar ratio of the components was cellulose–sodium hydroxide–epichlorohydrin, 1:1:0.5. The cellulose modified in this manner contained approximately 0.4–0.45 hydroxypropyl bridges per one D-glucose unit. The most suitable fraction (0.06–0.1 mesh) was obtained by sieving. The volume of gel swollen in water was 3.4 ml/g (ref. 7). The flow rate through a layer of 10 cm, at a pressure of 500 mm H_2O, was 150 ml/h cm^2.

S_E: the starch gel. This gel was prepared by cross-linking potato starch under alkaline conditions according to ref. 8. The volume of gel swollen in water was 2.5 ml/g and the flow rate through a 10 cm layer was 200 ml/h cm^2, at 500 mm H_2O pressure.

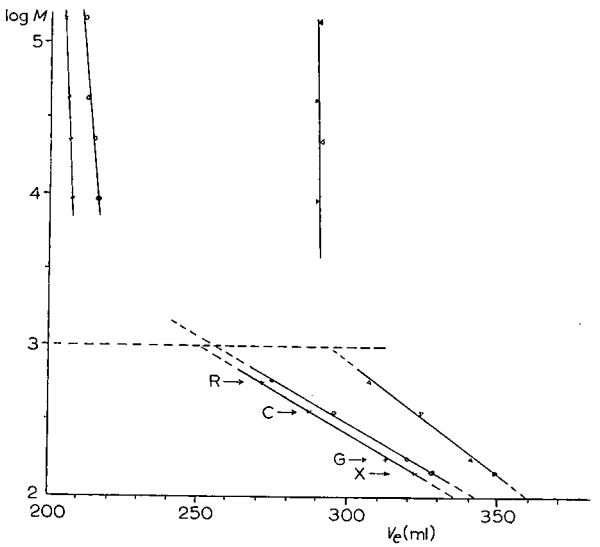

Fig. 1.Dependence of the molecular weight of oligosaccharides and dextrans upon elution volumes of various gels. Column 6 cm^2 × 78 cm. X = D-xylose; G = D-glucose; R = raffinose; C = cellobiose; + = Sephadex G-15; ○ = S_E; △ = C_E.

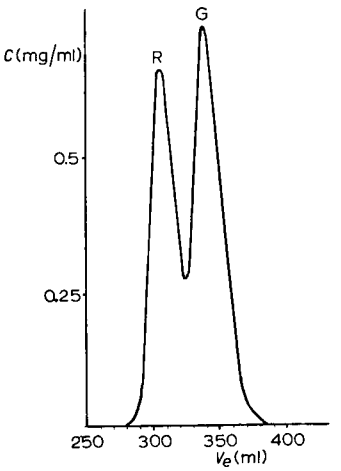

Fig. 2. Elution curve of a mixture of raffinose and D-glucose on C_E. Column 6 cm^2 × 78 cm. R = raffinose; G = D-glucose.

Sephadex G-15. A commercially available product of bed volume 3 ml/g, 0.04–0.12 mesh was used.

GPC equipment and the separated mixtures

The gels were tested in a simple apparatus[9] which comprised a glass column closed with a teflon piston. The concentration of the effluent was determined using a differential refractometer (Waters, Model R-4). The solvent was supplied by means of a Waters' pumping system. Column dimensions were 6 cm^2 × 78 cm and 6 cm^2 × 156 cm, respectively.

The columns were packed by allowing the swollen gel to settle at an absolute

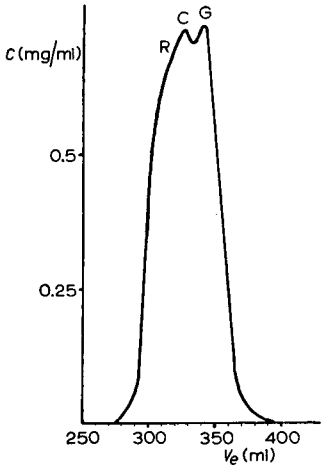

Fig. 3. Elution curve of a mixture of raffinose, cellobiose and D-glucose on C_E. Column 6 cm^2 × 78 cm. R = raffinose; C = cellobiose, G = D-glucose.

effluent flow rate two times larger than that at elution. Several series of experiments were carried out using D-glucose, D-xylose, cellobiose, raffinose and four fractions of dextran of mol. wt. 9300–167 000 (determined viscosimetrically). When the shorter column (length 78 cm and the total bed volume 478 cm^3) was used the sample was injected at a concentration of each of the components of 4 mg/ml either separately or in various combinations (Figs. 2–5). In experiments on the longer column (total bed volume 935 cm^3) the sample concentration was doubled. The volume of the injected sample was 2 ml in every case.

RESULTS AND DISCUSSION

In all experiments symmetrical elution curves were obtained. The gels were tested by plotting log M against elution volume V_e which was linear over a certain range (Fig. 1). As elution volume the one was taken at which the elution curve reached its maximum. The elution volumes remained constant regardless of the

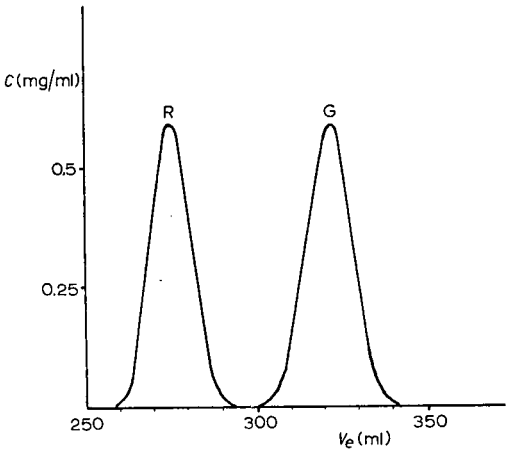

Fig. 4. Elution curve of a mixture of raffinose and D-glucose on S_E. Column 6 cm^2 × 78 cm. R = raffinose; G = D-glucose.

combination of components in the sample injected, *e.g.*, individual components, a mixture of two (raffinose and D-glucose, Figs. 2 and 4) or three components (raffinose, cellobiose and D-glucose, Figs. 3 and 5). These experiments were carried out on the 78 cm column.

It follows from the above that modified starch has the best separation properties over the range of molecular weight under investigation and obviously, also the most advantageous distribution of pores. As standard oligosaccharides of molecular weight in the range $7 \cdot 10^2 - 9 \cdot 10^3$ were not available, no conclusion as to the range over which the dependance log M *vs.* V_e is linear could be made. We presume, however, that extrapolation up to log $M \leqq 3.2$ may be done; *i.e.*, a satisfactory separation of oligosaccharides up to molecular weight $M \leqq 1200$ should well be possible.

The elution volume of high molecular weight dextrans, which can be considered virtually excluded from the gel pores, is given by the interstitial volume V_0, and thus it is useless for separation purposes. Its value depends both on the conditions of

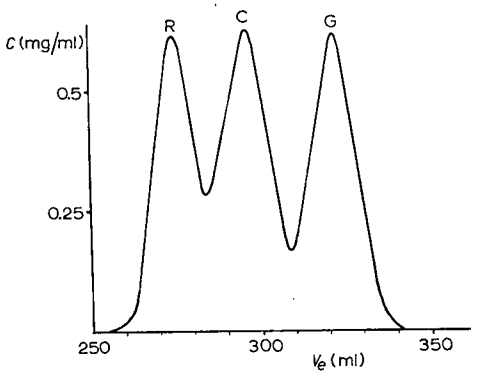

Fig. 5. Elution curve of a mixture of raffinose, cellobiose and D-glucose on S_E. Column 6 cm² × 78 cm. R = raffinose; C = cellobiose; G = D-glucose.

column packing and on the size and shape of the gel particles. As in the course of our work the experimental conditions remained unchanged, the shape of the particles turned out to be the determining factor. The comparative data are summarized in Table I where V_0 is expressed in per cent of the total bed volume V_t. It is obvious that the spherical particles of Sephadex G-15 give the lowest and rod-shaped cellulose the highest V_0.

Let us now compare the slopes of the linear parts of the calibration dependence log M *vs.* V_e for three different gels (Fig. 1). Lowest separation efficiency is shown by the cellulose gel while best results can be obtained on starch gel (*cf.* also Figs. 2–5).

The shape of the calibration dependence log M *vs.* V_e for starch, in the region of $4 < \log M < 5$, shows that here the large pore volume is not quite negligible.

TABLE I

INTERSTITIAL VOLUME OF GELS WITH DIFFERENTLY SHAPED PARTICLES

Gel	*Shape*	V_0 *(ml)*	V_0 *(%)*
Sephadex G-15	Spherical	204	43.6
Starch cross-linked with epichlorohydrin (C_E)	Irregularly oval, "potato-shaped"	212	45.3
Cellulose cross-linked with epichlorohydrin (C_E)	Rod-shaped	290	62.0

However, the selectivity of the gel over this range of molecular weights is very low. On the other hand, over the lower range of molecular weights, using an adequately long column, complete separation of the components under investigation was obtained (Fig. 6).

In Table II, some of our results are compared with those of MARTIN AND ROWLAND[4] obtained on non-modified decrystallized cellulose and on cellulose cross-linked with formaldehyde containing up to 5.2% of formaldehyde. Here we give the differences expressed in terms of reduced elution volume $V_{red} = V_e/V_t$ of two

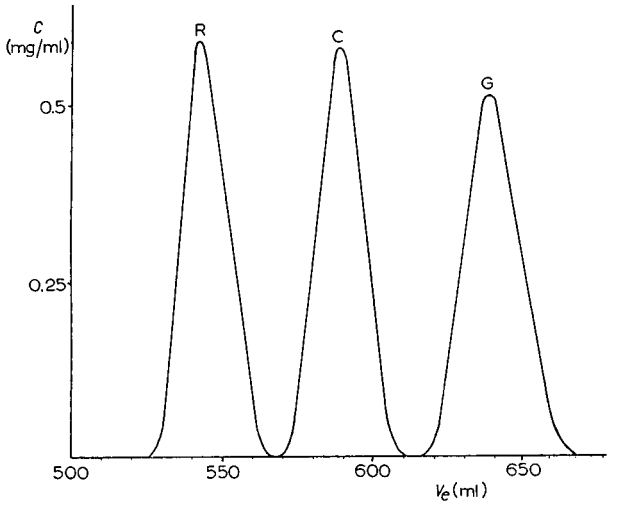

Fig. 6. Elution curve of a mixture of raffinose, cellobiose and D-glucose on S_E. Column 6 cm^2 × 156 cm. R = raffinose; C = cellobiose; G = D-glucose.

fictitious substances of mol. wt. 100 and 1000 obtained by extrapolation. Reduced elution volume, since it is independent of column size (as long as this is not extremely small) can be a useful criterion of comparison of the separation efficiency of individual gels.

Concluding, starch cross-linked with epichlorohydrin is well suited for sepa-

TABLE II

THE DIFFERENCES IN REDUCED ELUTION VOLUMES (V_{red})[a] OF SUBSTANCES WITH MOL. WT. = 100 AND 1000 AS FOUND FOR VARIOUS GELS.

Gel	$(V_{red})_{1000}^{100}$
Sephadex G-15	0.182
Starch cross-linked with epichlorohydrin (S_E)	0.188
Cellulose cross-linked with epichlorohydrin (C_E)	0.141
Cellulose cross-linked with formaldehyde[b]	0.138
Non-modified powdered cellulose[b]	0.076

[a] $V_{red} = V_e/V_t$, where V_e is elution volume and V_t the total volume.
[b] Calculation according to results of MARTIN AND ROWLAND[4].

ration of oligosaccharides, on the other hand the separation properties of a gel based on modified cellulose is not very good. This may, however, be associated with the larger interstitial volume V_0 caused by rod-shaped gel particles (Table I). The properties of C_E and those of cellulose cross-linked with formaldehyde[4], as shown in Table II, are approximately the same.

REFERENCES

1 P. B. G. FLODIN AND J. PORATH, *Nature*, 183 (1959) 1657.
2 P. B. G. FLODIN AND J. PORATH, *Nature*, 188 (1960) 493.
3 H. DETERMANN, H. REHNER AND T. WIELAND, *Makromol. Chem.*, 114 (1968) 263.
4 L. F. MARTIN AND S. P. ROWLAND, *J. Chromatogr.*, 22 (1967) 139.
5 G. H. LATHE AND C. R. J. RUTHVEN, *Biochem. J.*, 62 (1956) 665.
6 Ľ. KUNIAK AND B. ALINČE, *Czech. Pat.*, 132508, 14.III.1969.
7 Ľ. KUNIAK AND B. ALINČE, *Czech. Pat.*, 136062, 5.I.1970.
8 Ľ. KUNIAK AND P. LUBY, *PV 7193-70* claimed 23.10.1970, Czech. patent.
9 D. MCCALLUM, *Makromol. Chem.*, 100 (1967) 117.

CHROM. 5319

A METHOD FOR THE DETERMINATION OF THE MOLECULAR WEIGHT AND MOLECULAR-WEIGHT DISTRIBUTION OF CHONDROITIN SULPHATE

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SUMMARY

A simple micro-method for the determination of molecular weights of chondroitin sulphate has been developed. Columns of Sephadex G-200 and Sepharose 6B were calibrated with fractions of chondroitin sulphate, the molecular weights of which had been determined by independent methods. The use of the calibrated columns for determining the molecular weights and molecular-weight distributions of unknown samples of chondroitin sulphate by gel chromatography is described. The effects of variations in the ionic strength of the eluant were studied. For the average of duplicate determinations of the molecular weight ($\bar{M}_w$) the 95% confidence interval was $\bar{M}_w \pm 3\%$.

INTRODUCTION

With the development of gel chromatography[1], a useful tool for the separation of macromolecules became available. It has been extensively employed in preparative procedures and has also been used for analytical purposes[2]. The application of this technique for the molecular-weight determination of proteins has been reported by several authors[3–5]. Furthermore, the method has been successfully applied to the molecular-weight distribution analysis of polydisperse systems, including hydrophobic[6,7] as well as water-soluble polymers[8,9].

A large number of methods have been employed for the molecular-weight determination of chondroitin sulphate, one of the glycosaminoglycans of connective tissue. These methods include ultracentrifugation[10–13], osmometry[14,15], light scattering[14,16,17] and chemical analysis[15,18]. The various drawbacks of these techniques have emphasized the need for a new, simple, rapid and inexpensive method for small amounts of material.

In the present investigation a method based on the principle of gel chromatography has been developed for the molecular-weight and molecular-weight distribution analysis of chondroitin sulphate. The method has been outlined previously by WASTESON[19] and, independently, by CONSTANTOPOULOS *et al.*[20].

MATERIALS AND METHODS

The preparation and characterization of chondroitin sulphate fractions have been described in a previous publication[21]. Eleven essentially monodisperse fractions were obtained with molecular weights ranging from 2,400 to 36,000. Some relevant physicochemical data are recapitulated in Table I.

TABLE I

PHYSICOCHEMICAL DATA FOR FRACTIONS OF CHONDROITIN SULPHATE[a]

Chondroitin sulphate fraction No.	*K_{av} in 0.2 M NaCl*[b]		*Stokes' radius (r_s) in 0.2 M NaCl (Å)*[c]	*Molecular weight*[d]
	Sephadex G-200	*Sepharose 6 B*		
1	0.11	0.39	58.5	35,800
2	0.20	0.45	49.5	25,400
3	0.28	0.51	43.5	19,200
4	0.34	0.55	39.5	16,500
5	0.41	—	35.0	13,900
6	0.47	—	32.0	11,100
7	0.30	—	42.0	18,300
8	0.45	0.59	33.0	12,400
9	0.61	0.68	25.0	8,600
10	0.74	0.77	18.0	4,800
11	0.84	0.85	12.5	2,400

[a] See also Wasteson[21].
[b] Calculated according to LAURENT AND KILLANDER[29].
[c] Calculated by use of r_s values of Ficoll fractions, as described previously[21].
[d] Calculated as $\frac{1}{2}[\bar{M}_{w(s/D_{app})} + \bar{M}_{w(eq)}]$ (ref. 21), except for the value of fraction 7, expressing $\bar{M}_{w(s/D_{app})}$ only.

Three well-characterized fractions of Ficoll, No. XIII, XV and XVI with Stokes' radii 49.0, 34.5 and 26.4 Å, respectively, were available in our laboratory[22].

Chondroitin sulphate was isolated from various sources (see below) by digestion with papain[23] followed by precipitation of the liberated polysaccharide with cetylpyridinium chloride from 0.3 *M* NaCl[24]. ^{35}S-labelled chondroitin sulphate was prepared from slices of rat costal cartilage which had been incubated *in vitro* with [^{35}S]sulphate[25]. The specific activity of the preparation was approximately 1000 c.p.m./μg uronic acid.

Chondroitin-sulphate proteoglycan (PPL) was prepared from bovine nasal septa according to GERBER *et al.*[26]. Dextran gel (Sephadex G-200) and agarose gels (Sepharose 6B and 2B) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Uronic acid was determined by the carbazole method of BITTER AND MUIR[27] or by an automated modification of this method[28]. ^{35}S-activity was measured in a Beckman LS-250 liquid scintillation counter with a dioxane–naphthalene–PPO mixture as the scintillation medium.

Analytical gel chromatography on Sephadex G-200 was performed at 4° on a 0.8 × 63.5 cm column essentially as described previously[21]. Generally, 100–200 μg of chondroitin sulphate or 1 mg of Ficoll were applied to the column, which was

eluted with 0.2 *M* NaCl at a flow rate of about 3 ml·cm^{-2}·h^{-1}. The effluent volume was determined by gravimetry, and the effluent fractions (0.7–0.9 ml) were analysed for uronic acid or ^{35}S-activity.

Analytical gel chromatography on Sepharose 6B was carried out in a similar manner; in this case 200–300 μg of polysaccharide were applied to the column (1.3 × 79 cm).

The void volumes (V_0) and the total volumes (V_t) of the columns[29] were determined by chromatography of a high-molecular-weight fraction of dextran ($\bar{M}_w$ 12 × 10^6) and of tritiated water (Radiochemical Centre, Amersham, Great Britain), respectively[30,31].

The experimental conditions were also extended to include gel chromatography at different ionic strengths and at different concentrations of polysaccharide.

RESULTS

Chromatographic conditions

Effect of ionic strength. Considerable variation in the elution pattern was observed when a fraction of chondroitin sulphate (fraction 3, $\bar{M}_w$ 19,200 (ref. 21)) was chromatographed on the Sephadex column at different ionic strengths (Table II). At low ionic strength the polysaccharide emerged early in the eluate, indicating that the chondroitin sulphate molecules were extended. Also, at low ionic strength ($I < 1 \times 10^{-3}$) the chromatograms tended to become broad and distorted, especially when increased amounts of polysaccharide were applied to the column. With increasing ionic strength the elution of the polysaccharide was progressively retarded, indicating an increased coiling of the molecules. At $I > 0.4$ no further increase in K_{av} was noted. Qualitatively similar results have been obtained with heparin[32]. The variations in the elution pattern of chondroitin sulphate were not due to changes

TABLE II

CHROMATOGRAPHY OF FICOLL AND CHONDROITIN SULPHATE ON SEPHADEX G-200 AT DIFFERENT IONIC STRENGTHS

Ionic strength	K_{av}[a] *for*		r_s *for chondroitin sulphate fraction 3 (Å)*[b]
	Ficoll fraction XV	*Chondroitin sulphate fraction 3*	
5 × 10^{-5}	0.41	[c]	[c]
2 × 10^{-4}	0.42	0.14	55
5 × 10^{-4}	0.42	0.14	55
5 × 10^{-3}	0.41	0.15	53
5 × 10^{-2}	0.42	0.21	48
1 × 10^{-1}	0.42	0.24	46
2 × 10^{-1}	0.42	0.27	44
3 × 10^{-1}	0.41	0.28	43
4 × 10^{-1}	0.39	0.29	43
1 × 10^{0}	0.42	0.29	43

[a] Calculated according to LAURENT AND KILLANDER[29].

[b] Calculated by use of r_s values of Ficoll fractions, as described previously[21].

[c] At ionic strengths <1 × 10^{-4} the calculations of K_{av} and r_s of the chondroitin sulphate were precluded by the markedly irregular chromatographic behaviour of the latter.

of the properties of the gel, since the K_{av} values of Ficoll were not influenced by the ionic strength (Table II). An estimate of the size of the chondroitin sulphate molecules at the different ionic strengths (Table II) was obtained by comparison with Ficoll fractions of similar K_{av}, whose Stokes' radii had been established earlier. Apparently the radius of gyration of the chondroitin sulphate molecules (mol. wt. 19,200) was approximately 25% larger at very low ionic strength than at $I = 0.2$. It was concluded from these experiments that 0.2 M NaCl provides an adequate ionic strength for the chromatography of chondroitin sulphate; the effect of further increased salt concentration on the Stokes' radius was almost negligible. Therefore, the experiments described below were all carried out with 0.2 M NaCl as eluant, since at higher concentrations of salt the automated analysis of uronic acid was hampered by the formation of HCl gas.

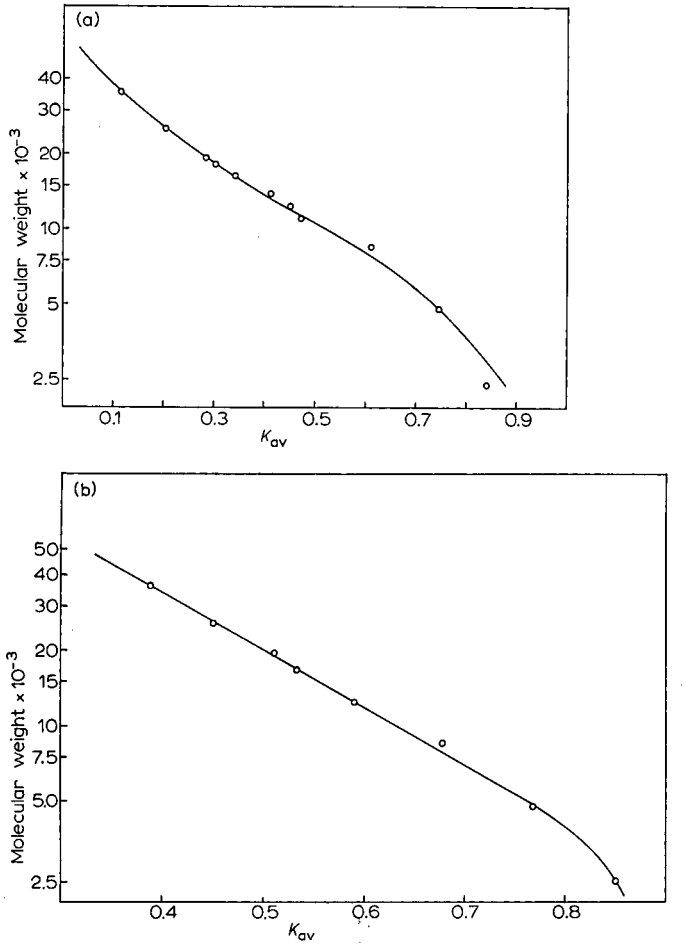

Fig. 1. Calibration of columns of Sephadex G-200 (a) and Sepharose 6B (b) with reference fractions of chondroitin sulphate. The molecular weights of the different fractions have been plotted *vs.* the respective K_{av} values.

Effect of polysaccharide concentration. Chromatographies of chondroitin sulphate on Sephadex were also obtained at different concentrations of the polysaccharide. A moderate increase in the polysaccharide concentration did not significantly affect the elution patterns. However, if more than 3 mg of chondroitin sulphate were charged on the column, corresponding to an increase in the concentration by a factor of about 20, the elution of the polysaccharide was retarded and the resulting profile distorted, indicating marked intermolecular interactions[33,34]. However, at the low concentrations generally used, such interactions were insignificant.

Calibration of gel columns. The K_{av} values of the reference fractions of chondroitin sulphate on the gel columns were determined from V_0, V_t and the respective elution volumes for the fractions[29], and were then plotted *vs.* the logarithms of the molecular weights of the respective fractions (Table I, Fig. 1). As can be seen from Fig. 1, slightly curved plots were obtained.

Determination of the molecular weight and molecular-weight distribution of chondroitin sulphate

The established relationship between the molecular weight and the elution position (K_{av}) of chondroitin sulphate was used for determining the molecular weights of unknown samples of the polysaccharide. The following procedure has been applied to the chromatography data[8]. The elution profile for the preparation was divided into a large number of segments (for practical reasons *e.g.* 20), the K_{av} values of which correspond to certain molecular weights, M_i, according to the calibration curve (Fig. 1). The relative amount of chondroitin sulphate, W_i, within each fraction was calculated and used to derive the weight-average ($\bar{M}_w$) and number-average ($\bar{M}_n$) molecular weights of the whole preparation according to the equations:

$$\bar{M}_w = \Sigma W_i \cdot M_i \tag{1}$$

$$\bar{M}_n = \frac{1}{\sum \frac{W_i}{M_i}} \tag{2}$$

The cumulative molecular-weight distribution (Fig. 2) may be obtained by summing the amount of eluted material from the tail end of the chromatogram, *i.e.* from $K_{av} = 1$ to $K_{av} = 0$ and plotting this parameter *vs.* the molecular weight, obtained from K_{av} using the calibration curve (Fig. 1). By differentiating this function, the corresponding differential molecular-weight distribution is obtained (Fig. 2).

Resolution

The resolution of the Sephadex G-200 was studied by the following control experiments. A preparation of ^{35}S-labelled chondroitin sulphate ($\bar{M}_w = 18{,}300$; $\bar{M}_n = 14{,}800$) was fractionated on the column (Fig. 3). A subfraction was selected from a portion of the chromatogram, where zone-spreading would strongly interfere with the calculation of the molecular dispersion, and was then rechromatographed on the same column. The mol. wt. (13,200) of the selected molecular population, as determined from its elution profile (Fig. 3), was only slightly higher than that expected from its original elution position (12,500). This result indicates that the shape and

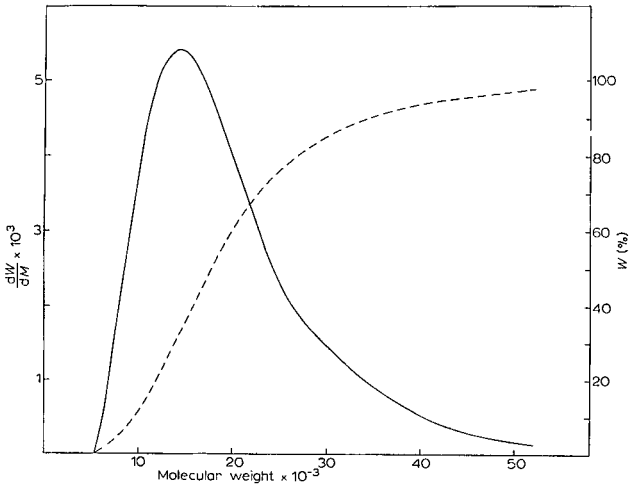

Fig. 2. The molecular-weight distribution of chondroitin sulphate from bovine nasal septum. The amount of eluted material has been accumulated from the tail end of the chromatogram (W) and plotted *vs.* molecular weight (- - - -). The total amount of material is taken as 100%. This curve has been differentiated to give the corresponding differential molecular-weight distribution function, dW/dM (———).

width of an eluted chondroitin sulphate peak essentially reflects the molecular dispersion of the preparation rather than a zone-broadening phenomenon. The validity of the method was also tested with two polydisperse chondroitin sulphate samples, composed of known amounts of well-characterized, practically mono-

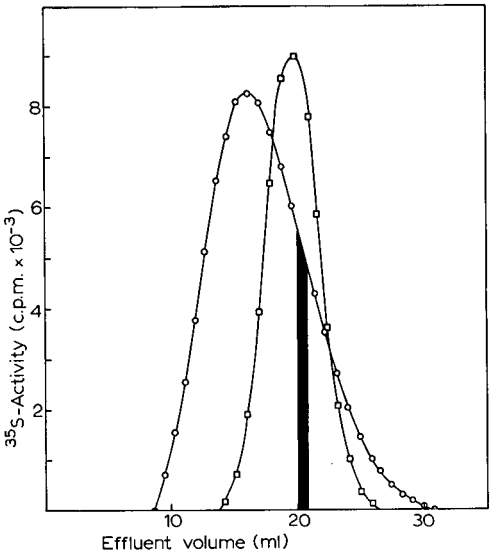

Fig. 3. Chromatography of ^{35}S-labelled chondroitin sulphate on a column of Sephadex G-200 (○—○). A subfraction corresponding to the shaded portion of the chromatogram was rechromatographed on the same column (□—□). The eluates were analysed for ^{35}S-activity. For comparison the activities of the rechromatographed sample were multiplied by a factor of 10.

disperse subfractions. One of these samples was a mixture, composed of equal amounts of fractions 2, 3, 4 and 5 (Table I). The $\bar{M}_w$ (19,800) and $\bar{M}_n$ (16,200) values of this sample were determined by gel chromatography on the Sephadex column. Although they were in fair agreement with those calculated from the molecular weights of the constituent fractions (Table I) according to eqns. 1 and 2 ($\bar{M}_w$ = 18,800; $\bar{M}_n$ = 17,800), the ratio $\bar{M}_w/\bar{M}_n$ was higher, which may be ascribed mainly to the zone spreading on the gel column. A hyaluronidase-treated material, identical with that used for the preparation of low-molecular weight fractions of chondroitin sulphate[21], was also examined. The molecular-weight values obtained by gel chromatography were $\bar{M}_w$ = 9,700 and $\bar{M}_n$ = 5,700, as compared to the theoretical values of 10,700 and 8,700, respectively. The $\bar{M}_w$ values were thus in fair agreement. The discrepancy between the $\bar{M}_n$ values remains unexplained, but may conceivably reflect some degree of molecular dispersity in the constituent fractions. However, an additional and probably more important factor is the error due to zone-spreading, which is most pronounced in the low-molecular-weight region, where the slope of the calibration curve is steep.

Reproducibility

The reproducibility of the analytical system was investigated by repeated chromatography of two preparations of chondroitin sulphate from chick-embryo epiphyseal cartilage and bovine nasal septum, respectively. The molecular weights ($\bar{M}_w$) of the two tested preparations, determined 17 and 10 times, were 27,700 and 19,800 and the corresponding standard deviations 0.8% and 0.7%, respectively.

For the average of duplicate determinations of the molecular weight ($\bar{M}_w$), a 95% confidence interval of $\bar{M}_w \pm 3\%$ is obtained[35].

Applications of the method

The molecular weights of a large number of chondroitin sulphate preparations have been determined by the present method. Most of this work, covering both structural and metabolic aspects of the chain length of chondroitin sulphate, will be presented elsewhere, and only a few applications will be mentioned here. The molecular weights of chondroitin sulphates from various sources are given in Table III.

Comparatively high molecular weights were noted for chondroitin sulphate prepared from compact bone of normal and rachitic puppies[36], in agreement with the findings of HJERTQUIST AND VEJLENS[37]. Since the material was eluted with or near the void volume of the Sephadex column, these preparations were analysed on the column of Sepharose 6B, which gives a superior resolution in the high-molecular-weight range to that obtained with Sephadex G-200. By comparison chondroitin sulphate isolated from epiphyseal cartilage of 14-day-old chick embryos and from bovine nasal septa showed considerably lower molecular weights (see also BUDDECKE *et al.*[17] and LUSCOMBE AND PHELPS[12]).

Using the present technique the molecular weight of chondroitin sulphate was also studied in relation to the macromolecular properties of the chondroitin-sulphate proteoglycan. Protein-polysaccharide, isolated from bovine nasal septa[26], was separated on a preparative column of Sepharose 2B into four fractions (I–IV) as indicated in Fig. 4. These fractions, the molecular weights of which were estimated to range from approximately 1×10^6 to approximately 10×10^6 (ref. 38) were then degraded

TABLE III

MOLECULAR WEIGHTS OF CHONDROITIN SULPHATE FROM DIFFERENT SOURCES, DETERMINED BY GEL CHROMATOGRAPHY

$\bar{M}_w$ = weight-average molecular weight; $\bar{M}_n$ = number-average molecular weight.

Origin of chondroitin sulphate	*Remarks*	$\bar{M}_w$	$\bar{M}_n$
Compact bone of puppies	Prepared from normal animals, aged 3 months	44,000	26,000
Compact bone of puppies	Prepared from rachitic animals, aged 3 months	42,000	26,000
Chick epiphyseal plate	Prepared from 14-day-old embryos	29,000	23,000
Bovine nasal septa	Prepared by direct proteolytic digestion of the tissue	19,800	15,500
Bovine nasal septa	Prepared from PPL I[a]	17,900	13,600
Bovine nasal septa	Prepared from PPL II[a]	18,200	14,000
Bovine nasal septa	Prepared from PPL III[a]	18,000	13,700
Bovine nasal septa	Prepared from PPL IV[a]	17,800	13,500
Bovine nasal septa	Hyaluronidase-digested[b]	9,700	5,700

[a] See text and Fig. 4 for explanation.
[b] See WASTESON[21].

by proteolysis to yield single chondroitin sulphate chains (see MATERIALS AND METHODS). As seen from Table III the molecular sizes of the chondroitin sulphate fractions thus obtained were identical, and, as expected, in fair agreement with that of chondroitin sulphate isolated from bovine nasal septa by direct digestion of the tissue. It was concluded that the considerable variation in size of the protein-polysaccharide preparation was not the effect of a varying chain length of the polysaccharide moiety. TSIGANOS AND MUIR[39] arrived at similar conclusions by applying a different technique to the protein-chondroitin sulphate of pig laryngeal cartilage.

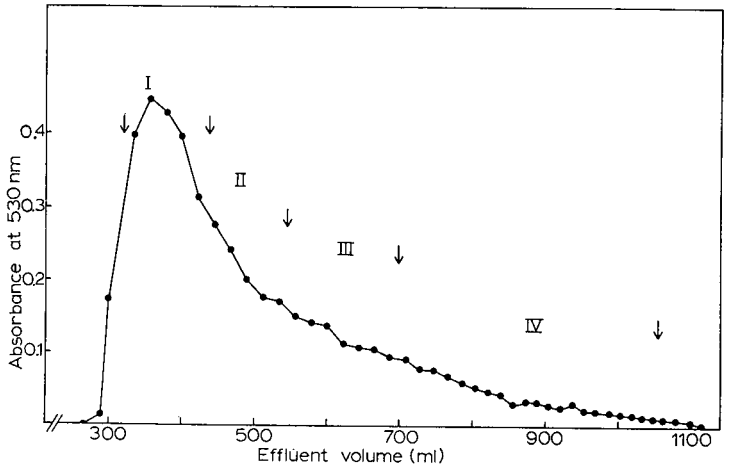

Fig. 4. Fractionation of PPL on a 4.2 × 84 cm column of Sepharose 2B. The effluent was analysed by the carbazole method and pooled into four fractions (PPL I–IV) as indicated by the arrows.

DISCUSSION

The present method for the determination of molecular weights by gel chromatography rests on the assumption that the elution position (K_{av}) of a compound is related to the molecular size. This relationship, which has been verified by several authors (for reviews see *e.g.* DETERMANN[2], JOHNSON AND PORTER[40], and LAURENT *et al.*[41]) may also be applied to the gel chromatography of chondroitin sulphate, as demonstrated by the calibration curves, shown in Fig. 1. Ideally, for a given gel, any K_{av} value should thus represent a certain molecular size. However, even minor variations in the experimental parameters may influence the elution behaviour, and, consequently, the experiments should be standardized as far as possible. Significant errors may thus arise from the use of too low an ionic strength (Table II) or from overloading of the column. The influence of temperature and flow rate has been discussed elsewhere[7,31,42].

The maintenance of proper experimental conditions is a prerequisite for the optimal performance of the gel column. However, even under optimal conditions chromatography of homogeneous molecular populations will yield peaks of finite widths. The significance of this zone-broadening phenomenon for the resolution of the analytical system has been discussed by several authors[40,43,44] and various corrective measures have been suggested[7,40,45,46]. The separation on a gel column due to difference in molecular size is proportional to the column length, whereas the zone spreading is proportional only to the square root of the column length. In consequence, the relative effects of zone spreading will decrease with increasing column length, as verified experimentally by WINZOR *et al.*[42,46]. In view of these considerations the column dimensions used in the present study would seem to be satisfactory. Nevertheless, an attempt was made to assess the effects of zone spreading in our system by rechromatographing a previously separated subfraction of chondroitin sulphate (Fig. 3). The resulting chromatogram showed that, for practical purposes, the effect of zone spreading could be neglected.

The resolving power of the analytical system was tested by chromatographing mixtures of well-characterized chondroitin sulphate subfractions (combined fractions 2–5 and 7–11, respectively). The results of these experiments demonstrated that the $\bar{M}_w$ and $\bar{M}_n$ values of polydisperse preparations of chondroitin sulphate may be adequately determined by the gel chromatography method. However, care should be taken in evaluating $\bar{M}_n$ values of chondroitin sulphate preparations eluted close to V_t, since the $\bar{M}_n$ value obtained for the hyaluronidase-treated preparation was probably too low. For samples of low dispersity, however, the reliability is probably sufficient for most biological problems, since the theoretical and experimental $\bar{M}_w$ values were in reasonable agreement.

Although the resolving power of the gel columns is optimal only within a limited fractionation range, the separation properties of Sephadex G-200 adequately cover the molecular-weight range of chondroitin sulphate[41]. While most chondroitin sulphates have molecular weights less than 30 000, some species, *e.g.* from bone, penetrate the upper limit of the separation range for Sephadex G-200. Therefore, for the high-molecular species of chondroitin sulphate, Sepharose 6B is preferred, since it is more permeable to large macromolecules than in Sephadex G-200. It should

be pointed out, however, that the overall resolution of Sepharose 6B is somewhat inferior to that of Sephadex G-200 (ref. 41).

The detailed characterization of chondroitin sulphate specimens, isolated from small amounts of tissue, is often limited by the supply of the polysaccharide. The small amounts of material required by the present method is one of the main advantages in relation to conventional methods for the determination of molecular weights, such as osmometry, light scattering or even ultracentrifugation. The relative simplicity and inexpensiveness of the analytical system further enhances the applicability of the present technique in relation to others. Finally, it should be noted that gel chromatography offers unique possibilities in metabolic studies for determining selectively the molecular weights of radioactively labelled polysaccharides.

The main drawback of the gel chromatographic method lies in the preparation and characterization of the reference substances required for the calibration curve. The necessity of using homologous preparations as references has been pointed out previously: the exclusion properties of a molecule on a gel column depend primarily on the size and form of the molecule and only indirectly on its molecular weight[41]. This is demonstrated by the relatively low K_{av} of chondroitin sulphate as compared to its molecular weight. Thus serum albumin (molecular weight 69 000) has the same K_{av} on Sephadex G-200 as a chondroitin sulphate with a molecular weight of 13,000. Further it should be borne in mind that variations in the properties of the gels and in the mode of operation may preclude the direct application of a calibration curve, obtained in a different laboratory.

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REFERENCES

1 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
2 H. DETERMANN, *Gel Chromatography*, Springer, New York, 1968.
3 P. ANDREWS, *Biochem. J.*, 96 (1965) 1595.
4 L. M. SIEGEL AND K. J. MONTY, *Biochim. Biophys. Acta*, 112 (1966) 346.
5 W. W. FISH, K. G. MANN AND C. TANFORD, *J. Biol. Chem.*, 244 (1969) 4989.
6 J. C. MOORE, *J. Polym. Sci.*, A2 (1964) 835.
7 J. C. MOORE AND J. G. HENDRICKSON, *J. Polym. Sci.*, C (1965) 233.
8 K. A. GRANATH AND B. E. KVIST, *J. Chromatogr.*, 28 (1967) 69.
9 S. A. BARKER AND N. M. YOUNG, *Carbohyd. Res.*, 2 (1966) 363.
10 H.-G. ELIAS AND F. PATAT, *Abhandlungen der Mathematisch-Naturwissenschaftlichen Klasse*, No. 4, Verlag der Akademie der Wissenschaften und der Literatur, Mainz, 1960, p. 223.
11 E. MARLER AND E. A. DAVIDSON, *Proc. Natl. Acad. Sci.*, 54 (1965) 648.
12 M. LUSCOMBE AND C. F. PHELPS, *Biochem. J.*, 103 (1967) 103.
13 H. GREILING, H. W. STUHLSATZ AND L. PLAGEMANN, *Z. Physiol. Chem.*, 348 (1967) 121.
14 M. B. MATHEWS, *Arch. Biochem. Biophys.*, 61 (1956) 367.
15 S. H. PARTRIDGE, H. DAVIS AND G. S. ADAIR, *Biochem. J.*, 79 (1961) 15.
16 R. V. WEBBER AND S. T. BAYLEY, *Can. J. Biochem. Physiol.*, 34 (1956) 993.
17 E. BUDDECKE, W. KRÖZ AND E. LANKA, *Z. Physiol. Chem.*, 331 (1963) 196.

18 H. M. MUIR, in G. QUINTARELLI (Editor), *The Chemical Physiology of Mucopolysaccharides*, Little and Brown, Boston, 1968, p. 1.
19 Å. WASTESON, *Biochim. Biophys. Acta*, 177 (1969) 152.
20 G. CONSTANTOPOULOS, A. S. DEKABAN AND W. R. CARROLL, *Anal. Biochem.*, 31 (1969) 59.
21 Å. WASTESON, *Biochem. J.*, 122 (1971) 477.
22 T. C. LAURENT AND K. A. GRANATH, *Biochim. Biophys. Acta*, 136 (1968) 191.
23 L.-Å. FRANSSON AND L. RODÉN, *J. Biol. Chem.*, 242 (1967) 4161.
24 J. E. SCOTT, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 8, Interscience, New York, 1960, p. 145.
25 Å. WASTESON AND U. LINDAHL, unpublished results.
26 B. R. GERBER, E. C. FRANKLIN AND M. SCHUBERT, *J. Biol. Chem.*, 235 (1960) 2870.
27 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.
28 E. A. BALAZS, K. O. BERNTSEN, J. KAROSSA AND D. A. SWANN, *Anal. Biochem.*, 12 (1965) 547.
29 T. C. LAURENT AND J. KILLANDER, *J. Chromatogr.*, 14 (1964) 317.
30 N. V. B. MARSDEN, *Ann. N. Y. Acad. Sci.*, 125 (1965) 428.
31 B. ÖBRINK, T. C. LAURENT AND R. RIGLER, *J. Chromatogr.*, 31 (1967) 48.
32 M. SKALKA, *J. Chromatogr.*, 33 (1968) 456.
33 D. J. WINZOR AND L. W. NICHOL, *Biochim. Biophys. Acta*, 104 (1965) 1.
34 K. HELLSING, *J. Chromatogr.*, 36 (1968) 170.
35 A. HALD, *Statistical Tables and Formulas*, John Wiley & Sons, New York, 1952.
36 B. ENGFELDT AND Å. WASTESON, unpublished results.
37 S.-O. HJERTQUIST AND L. VEJLENS, *Calc. Tiss. Res.*, 2 (1968) 314.
38 B. ÖBRINK, personal communication.
39 C. P. TSIGANOS AND H. M. MUIR, *Biochem. J.*, 113 (1969) 885.
40 J. F. JOHNSON AND R. S. PORTER, *Analytical Gel Permeation Chromatography, J. Polym. Sci.*, Part C, Polymer Symposia, 1968.
41 T. C. LAURENT, B. ÖBRINK, K. HELLSING AND Å. WASTESON, in TH. GERRITSEN (Editor), *Modern Separation Methods of Macromolecules and Particles*, John Wiley & Sons, New York, 1969, p. 199.
42 G. E. HIBBERED, A. G. OGSTON AND D. J. WINZOR, *J. Chromatogr.*, 48 (1970) 393.
43 J. C. GIDDINGS, *Dynamics of Chromatography*, Vol. I, Marcel Dekker, New York, 1965, p. 1.
44 J. C. GIDDINGS, *Anal. Chem.*, 40 (1968) 2143.
45 L. H. TUNG, *J. Appl. Polym. Sci.*, 10 (1966) 375.
46 N. W. H. CHEETHAM AND D. J. WINZOR, *J. Chromatogr.*, 48 (1970) 400.

CHROM. 5378

A TWO-DIMENSIONAL SCANNER FOR RADIOCHROMATOGRAMS EMPLOYING AN ELECTRON MULTIPLIER DETECTOR *IN VACUO*

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SUMMARY

A step scanner for the measurement of both one- and two-dimensional distributions of radionuclides in paper or thin-layer chromatography has been constructed and tested. Different versions of a miniaturized channel electron multiplier have been applied as detectors. A description is given of the principle of operation for this detector type, the vacuum scanner, and the way of data presentation.

Performance tests have been made with calibrated standards of ^{3}H and ^{14}C in the form of poly(methyl methacrylate) pieces with known electron emission defined as the number of electrons emitted per second per cm^2. Several thin-layer media have been used, mostly with directly applied spots of [^{3}H]- or [^{14}C]glucose solutions. Upon drying, spots of activities down to 5 nCi could easily be detected (spot areas were approximately 0.7 cm^2). A resolution of 1 mm could easily be achieved in the one-dimensional scans.

Some more results of a somewhat preliminary nature are reported. Some possible alternatives for the recording of measurements which use the channel electron multiplier detector are mentioned.

The use of scanning by electron multipliers as a method for radiochromatography has the advantages that the sample is neither damaged nor contaminated compared with conventional techniques which, for example, employ the admixture of wet (or dry) scintillators. It is felt that these advantages for certain applications more than compensate for the fact that the technique requires a vacuum of $5 \cdot 10^{-4}$ torr.

INTRODUCTION

In principle, a number of methods exist for radionuclide detection in chromatographic samples. In practice, however, the scintillation methods are still the most sensitive ones. In some cases there might nevertheless be a need for a system of detection which would leave the chromatogram undamaged and free from scintillator contaminations.

The purpose of this paper is to draw attention to a new type of electron detector

which may have some advantages over gas-flow GM detectors for the radiochromatographic detection of tritium (^3H). The discussion is in general terms and rather short, as the system is still under investigation. While further development of the described system seems possible, there are some obvious disadvantages. A preliminary description of the system has been given earlier[1].

For the detection of radionuclides other than ^3H and perhaps ^{14}C, the sensitivity of ordinary gas-flow Geiger Müller (GM) detectors is sufficient. For ^3H detection, on the other hand, the lower energy of the electrons results in path lengths of only a few μm in the sample substances or a few mm in air (or in the counter gas). This is the explanation for the low counting efficiency for tritium (the proportion of counts per disintegration is only about 0.5–5%). In addition, a preliminary (unpublished) experiment showed a slightly increased ^3H-electron exposure of X-ray films when exposed in a moderate vacuum. These considerations suggest the use of an electron multiplier detector operated in a vacuum system.

It was also supposed that the reproducibility of such a system would be much less dependent upon the maintenance of a constant gap between the detector and the chromatograms. This was found to be correct. The vacuum requirement, however, excludes the investigation of substances which are too volatile.

In GM tubes, a signal amplification is achieved as the single incoming electron starts a cascade of electrons which, on their path towards the central collector electrode, held at a positive h.t., collide with gas atoms which in their turn, upon ionization, give off new electrons, thus steadily increasing the cascade. In this way single electrons reaching the detector from the spots of the radiochromatogram may be counted or, alternatively, a smoothed current may be recorded.

For the class of particle detectors termed electron multipliers, the required signal amplification is achieved by successive impingements of electrons on to secondary emitting surfaces or dynodes, held at increasingly higher potentials. Eventually, the electron cascade is collected at an anode. Again, pulse counting and current measurement are alternative possibilities.

For the described detectors to work adequately, the gas pressure must be held equal to or below $5 \cdot 10^{-4}$ torr. This is partly because unwanted effects occur if positively ionized gas molecules in appreciable numbers drift backwards in the multiplier tube. Corona discharges must also be avoided. Therefore, the chromatogram scanner had to be enclosed in a vacuum chamber together with the detector and the chromatogram. Several constructional details stem from this requirement.

METHOD

Fig. 1 shows a diagram of the β-detection equipment. The chromatogram is supported by a 20 × 20 cm table which can be moved in two perpendicular directions by means of pulse-controlled step motors. The scanner is contained in a stainless steel housing which may be evacuated to 10^{-6} torr by means of an ordinary oil diffusion pump. The signal from the detector is amplified and recorded. In two dimensions a modified x–y recorder might be used. The position reproducibility is of the order of 0.5 mm. The signal, in the form of amplified pulses, may be recorded either as a d.c. output signal from a pulse count-rate meter, or in the form of a histogram by use of a special scaler. In the latter case, the height of each column is

proportional to the number of counts obtained in the corresponding position during the constant pre-set step time. Fig. 2 shows the vacuum scanner and the detector. Fig. 3 shows the whole experimental set-up. In the background (on the top of the rack) two units producing driving pulses for the step motors, which can be driven at various rates, can be seen. On top of these units is the h.t. power supply for the detector. One of the step motors with its reduction gear can be seen mounted on the left side of the chamber. The rotating shaft is coupled into the chamber through a vacuum seal. The electrical leads are taken through a vacuum seal situated on the

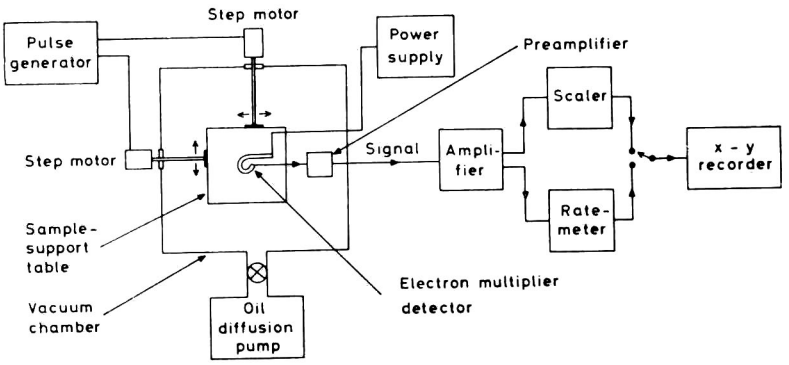


Fig. 1. Block diagram of the β-detection equipment. The step motor control unit and the electronic coupling of the recorder position to the detector position are not shown.

top of the chamber along with the Pirani gauge head. The vacuum valves and the diffusion pump are hidden from sight on the right-hand side of the chamber. At the bottom of the photograph is the Philips rate meter which was used and in front of this is the x–y recorder. Beside this is situated the electronic equipment of the Pirani gauge. Behind this the rotary pump is just visible. The special scaler, depicted in Fig. 1, is not shown here.

The vacuum system

The volume of the vacuum chamber is about 75 l. The walls are exclusively made from stainless steel and are of the order of 2 mm thick. A short tube of 10-cm bore connects the chamber to the oil diffusion pump via a baffle valve. A liquid nitrogen oil trap was planned but has been omitted without the introduction of complications. The pumps used are the "Speedivac" vapour diffusion pump model E 02 and "Speedivac" ES 150 high vacuum pump from Edwards High Vacuum Ltd., Sussex, Great Britain.

In the conventional manner, provisions were made for roughing the chamber by direct connection of the rotary pump during the initial phase of pumping. An Edwards Pirani gauge was used to measure the residual pressure in the chamber. One drawback was that the ionization gauge head could not be connected for measurement during the operation of the electron multiplier detector as the electrons produced by the Pirani head give an extremely large background count rate.

The mechanical units within the chamber were produced free from any large obstructed volumes and were made of materials that required a negligible degassing

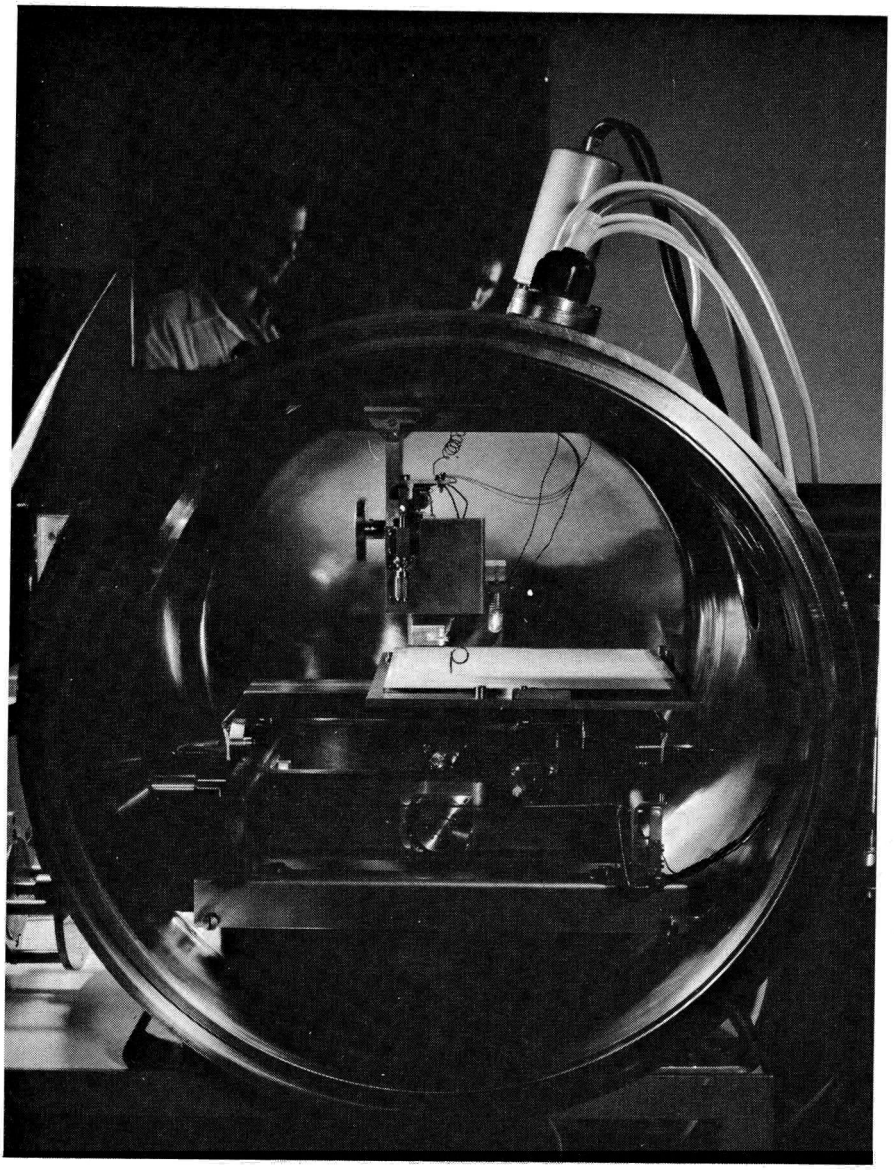


Fig. 2. The electron detector, closely coupled to its preamplifier, may be adjusted in height above the chromatogram which is seen fixed to the three-point support table of the vacuum x–y scanner.

period, after air admittance. During normal use, the required vacuum of $5 \cdot 10^{-4}$ torr can be reached in less than 15 min.

The scanning system

Two step motors are mounted outside the vacuum chamber with driving shafts coupled vacuum-tight through the chamber walls. An approximately 10:1 reduction gear is used. Inside the chamber, the moving table, 20 × 20 cm, can be drawn back

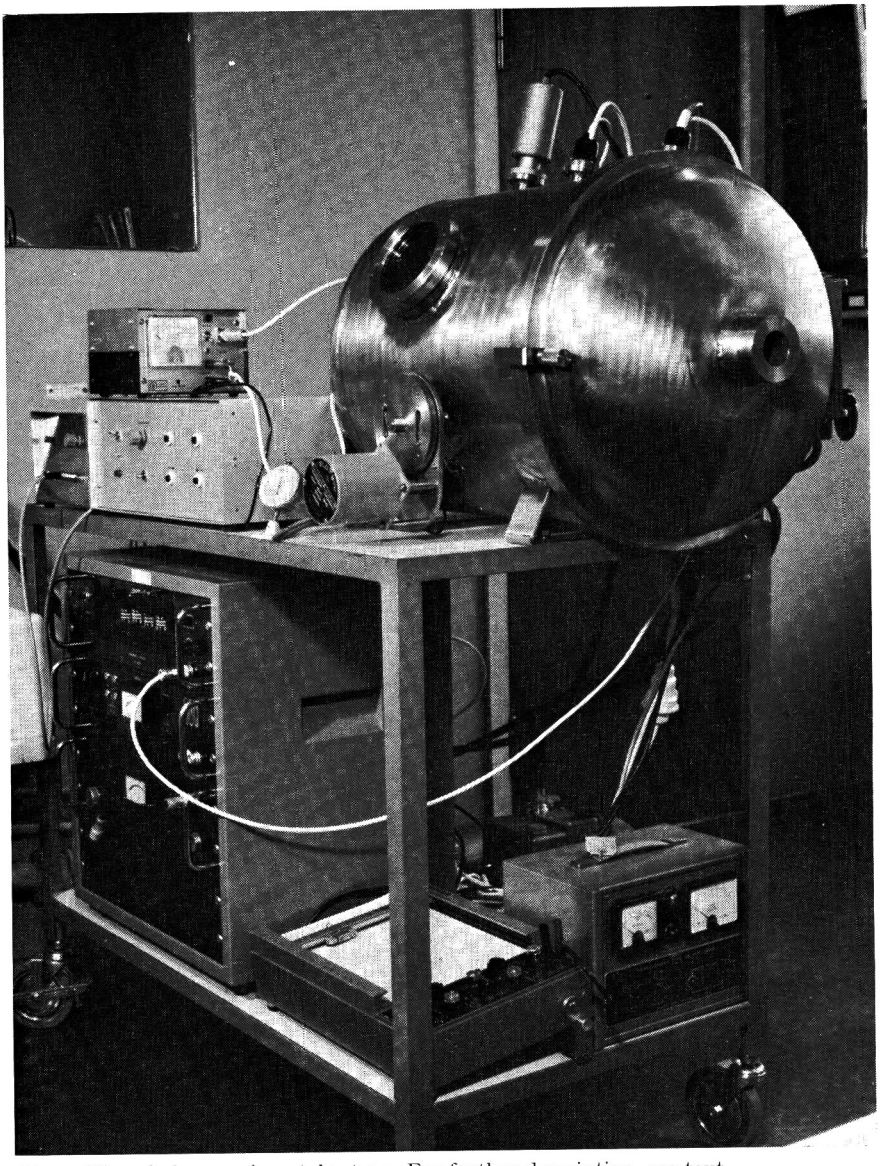


Fig. 3. The whole experimental set-up. For further description, see text.

and forth through 20 cm in two perpendicular directions by means of piano wire connected to rollers on the rotating shafts.

The motors are activated to move stepwise by means of electric pulses. Each step corresponds to *ca.* 0.3 mm table movement. Thus, a pulse generator is needed to produce the table movements. Some simple logic was added to perform automatic table operations. Using push-buttons, the table can be moved back and forth both in and perpendicular to the scan direction. Thus one can manually position the detector over a spot maximum and then start counting. In the automatic mode,

microswitches actuate end stops in both the scan and the return directions. During the return of the table, a sideways movement of a selected number of steps is performed. In this way, an automatic scanning of a two-dimensional chromatogram may be made. This operation imposes some requirements on the data presentation method which are discussed in a later section. It is also possible to add the counts obtained during two or four steps in the forward direction. This enables one to make a fast scan to localise the spots, which may be counted later. The scan system thus performs fast movements in short steps and then the table rests in this position for a specific time which may be preselected as one of eight possible counting intervals. The available numbers of steps per min are 1, 2, 4, 6, 12, 30, 60 and 120.

Detector types

Many types of electron multiplier are now available. They are all based on the principle of electron cascading, either within a structured system of dynodes or along a single high-resistance dynode film supported by an insulating material. The factor of multiplication or gain is given very roughly as $G = n^m$, where n is the number of secondary electrons emitted from the dynode surface per incident (primary) electron, and m is the number of multiplying jumps along the surface of the dynode strip (or simply number of dynodes in the first type). G may be as high as 10^6 (or 10^8 in the first types). We have applied miniaturized detectors of the continuous dynode type.

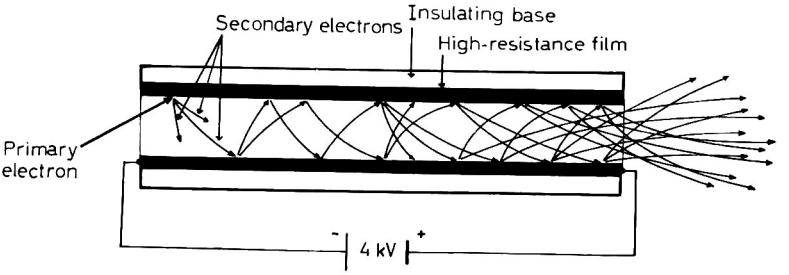


Fig. 4. Channel electron multiplier.

The channel electron multiplier is so called because the multiplying process takes place within a tube or channel. The first proposal for such a device was made by FARNSWORTH[2] in 1930. An electron cascade could, in principle, be produced by secondary emission from the internal wall of a tube of a resisting material, the ends of which are held at different potentials. An electron (or another particle) entering the low-potential end of the tube releases one or a few secondary electrons when striking the wall, as shown in Fig. 4. The secondary electrons are drawn towards a more positive potential and strike the opposite wall after some acceleration has taken place. New secondary electrons are released and the process continues along the full length of the tube. Contrary to conventional electron multipliers, which have an integral number of dynodes, the channel multipliers with their single continuous dynode strip have an indeterminate number of multiplication stages. In practice, the possibility of miniaturization is unique to the channel multipliers.

There is no evidence in the literature that FARNSWORTH succeeded in producing his suggested channel electron multiplier[3]. Thirty years elapsed while the technology of producing suitable resistive wall materials was developed. In 1960, OSCHEPKOV[4] and in 1962 GOODRICH AND WILEY[5] described working multiplier tubes and since about 1965 several experimental versions have been available from Mullard Ltd. The earliest available data were given by ADAMS AND MANLEY[3].

While these multipliers may be of the linear type shown in Fig. 4, they may take a variety of forms, provided that the length of the tube is kept equal to or larger than thirty times the tube diameter. It may be found for linear multipliers[3] that the gain is pressure-dependent owing to the feedback of positive ions which cause after-pulsing to occur. By avoiding the linear geometry, the pressure-dependence of the gain is removed[3].

The small dimensions of such detectors might be of importance when high spatial resolution is required. Likewise, the small dimensions of the counter are thought to be of importance for the total noise level. This has contributions from the radioactivity in wall materials, background radiation and thermally released electrons

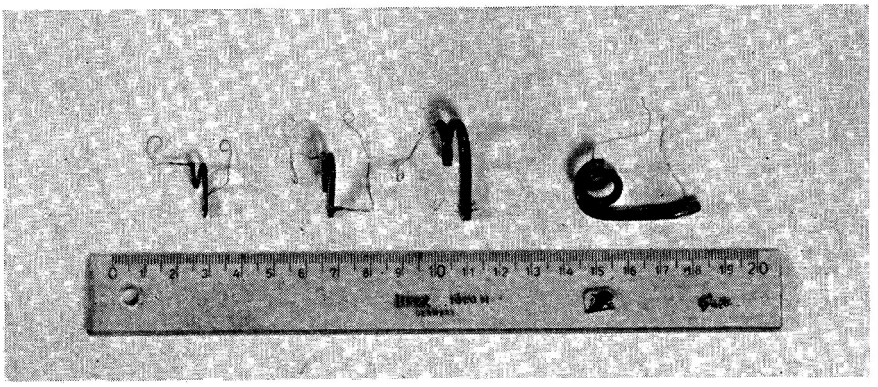


Fig. 5. Detectors. The right hand one is a side-slit detector with slit dimensions 1 × 5 mm. In addition, we used one with a 15-mm-long side-slit (not shown).

from the dynode coating. Mean noise count-rates less than 1 count per 10 min have been found in some detectors with no background screening other than the walls of the vacuum container made from 2-mm stainless steel.

For one-dimensional scanning, we obtained from Mullard Ltd. two specially made detectors having a side slit instead of the standard end opening. One of these detectors is shown in Fig. 5 together with the standard versions. The end of the tube had to be tilted upwards so that the electrode connection did not intersect the plane of the radiochromatogram. The other side slit counter had a planar geometry and a 1 × 15 mm slit.

For two-dimensional scans, detectors with an end opening are the most useful. These have a straight cathode end with a circular opening like that shown in Fig. 4 and a curved anode end as may be seen in Fig. 5. We have used detectors with opening diameters 2.0, 1.2 and 0.7 mm. The smallest apertures might be best suited for high resolution scans, but at the same time the electron collection area is diminished, so that the resulting count rates are also low.

We thought it very important that the detector should scan very close to the surface of the chromatogram, so as to cover the greatest possible solid angle of electron emission from the spots. However, as indicated later, this requirement is not as strong as one might think.

Electronics

Charge pulses containing about 10^6 electrons are not very difficult to detect. However, a preamplifier and pulse shaper was coupled close to the detector. A positive h.t. of about 4 kV is applied to the anode end of the multiplier. The negative pulses are amplified and either counted in a special scaler, which will be mentioned below, or fed to a rate meter from which the corresponding d.c. signal can be recorded. However, the stepped motion of the scanner makes the former version the most suitable.

Data presentation

To obtain the most informative presentation of radiochromatographic data, the various possible methods should preferably be tested in practice. In this respect, however, our investigation must as yet be considered somewhat preliminary. The one-dimensional display systems are naturally very much simpler than the two-dimensional ones and in the first instance we shall concern ourselves with approaches of the former type.

In one dimension, the radiochromatographic strips may be scanned by rate meter recording of the variation of the count rate along the activity profile. This mode of operation is not very suitable because, as mentioned earlier, the scan must be carried out stepwise. For each 0.3 mm along the track, one will see a pen movement around some mean value of count rate with deflections being large for small time constants (RC)* and small for large time constants. Between these vertical "bars" there will be 0.3-mm-long horizontal "connections".

A more useful type of display is achieved in the form of activity histograms if one uses a special scaler connected to the detector. Our scaler counts for the preselected counting period and then at the end actuates the plotting of an x–y recorder whose y-position is determined by a d.c. voltage proportional to the count number of the scaler, then the scaler is zeroed, the radiochromatogram table moved one step to the next position, and the detector starts counting again. The counting capacity of the scaler can be selected as either 10^2, 10^3 or 10^4 counts. The same pulse generator controls the step motor for advancement of the chromatogram and the plotting of the count number. The procedure is repeated until the end stop of the chromatogram support table is reached. The pen movement along the x-axis is controlled by a ten-turn potentiometer connected to the drive shaft from the step motor of the scanner. Normally it is most suitable to obtain the activity profile with a 1:1 x-axis scale. The reproducibility of the x-position of the recorder is about 0.5 mm.

While the scaler histograms obtained in the counting mode are not distorted at fast scanning speeds, this effect may show up when the rate meter mode is applied. Here the integration time is determined by the time constant which must be suitably chosen. If the time constant is too large for the scanning speed applied, the spot profiles may be distorted. This is illustrated in Fig. 6. Part (a) shows a scaler histo-

* R = resistance; C = capacitance.

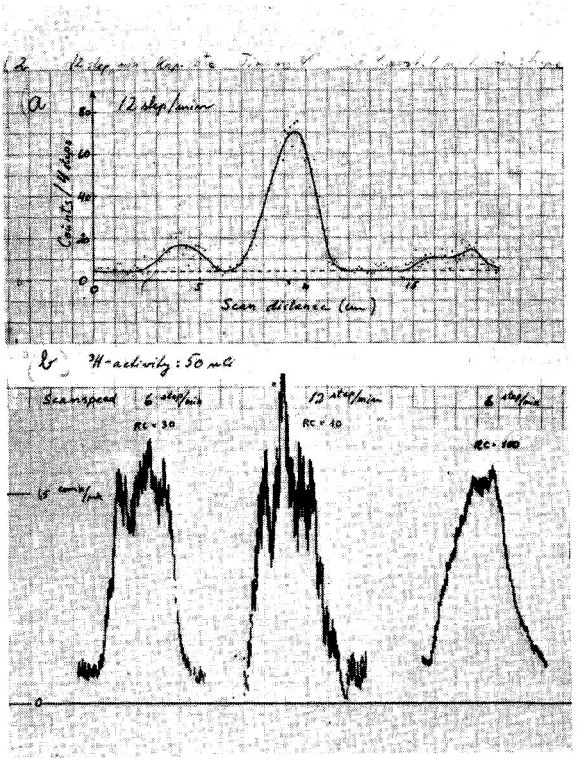


Fig. 6. Two ways of recording the scanned activity profiles. In part (a) a smooth curve has been drawn on the basis of a histogram plot where each dot indicates the number of counts reached in the preceding scan interval. Part (b) shows three examples of ordinary rate meter scanning of one and the same radiochromatogram spot. The scan speed and the time-constant of the rate meter have been varied. RC = 100 sec obviously is a too large time-constant as the profile has been tilted.

gram plot (by x–y recorder) and part (b) shows various rate meter-recorded spot profiles.

It would be an easy task to obtain a series of scaler print-out numbers instead of, or in addition to, the recording mentioned above.

The scanning of radiochromatograms where separation has been performed in two perpendicular directions is far more complicated. However, our scanner is suitable for this type of scanning. The whole chromatogram (20 × 20 cm) will, in the "auto" mode, be covered by parallel scans, the separation of which may be preselected. The difficulty is to find a useful way of presenting the activity readings.

One possibility would be to draw histograms, perhaps in different colours, along each track. However, this would probably result in a confusion of curves.

We have tried a different approach. Another paper[6] will describe the construction of a multisign plotter (fourteen different signs, seven in each of two colours) which may be mounted on the x–y recorder. In each position along the parallel tracks, count numbers may then be classified as belonging to one of fourteen consecutive ranges (with either a linear or a logaritmic scale) and the corresponding sign plotted. With a proper choice of track width, counter scale and sign types, the resulting

activity chart might be intelligible. The scaler with an output in the form of fourteen different activation signals for the plotter is not yet available. Its construction, however, poses no unsurmountable problems.

It is, of course, possible to construct an iso-activity chart manually from a series of parallel scans and to get approximate total spot activities (from standardization experiments) for a radiochromatogram. An example of an iso-activity chart obtained in this way is given below.

It is an obvious step to consider the use of a computer to assist in the drawing of charts and evaluation of spot activities. Count numbers could be recorded directly on magnetic or paper tape as they are made, and the resulting tape used as input to a computer. We already have the equipment necessary for the direct recording of measurements onto paper tape, but a suitable computer programme still has to be written.

PERFORMANCE TESTS

We shall now report some results obtained from tests made with various miniaturized detectors. It is of interest to investigate the behaviour of parameters such as counting efficiency, dependence upon the distance from the chromatogram, minimum spatial resolution, minimum detectable activity, etc.

These parameters are partly specific for each of the detectors but still of direct importance for the performance of the whole system. However, all parameters have not been investigated for all detectors.

Test materials

The measurements have been carried out partly on ^{3}H and ^{14}C standards, in the form of thin plates of poly(methyl methacrylate), the electron fluxes of which have been calculated by the producer (The Radiochemical Centre, Amersham, Great Britain). In addition several thin-layer media have been used with active glucose solution applied directly as circular spots. Such experiments, where the activities can be easily controlled, are the most suitable ones for testing of the performance of the scanner. In addition, a few measurements have been made on one- and two-dimensional radiochromatograms, of the paper or thin-layer type (either cellulose, silica gel or alumina).

Solutions of various concentrations of [^{3}H] and [^{14}C]glucose were made up from standard solutions, available from The Radiochemical Centre, Amersham. Useful spot activities were made for the diverse tests by pipetting small volumes (5 μl) of the various sugar solutions onto Whatman chromatographic papers or thin layers of adsorbent materials. Spot areas ranged from 0.3 to 1.1 cm^{2}.

Even though the spots tended to show drying rings when applied to dry samples of adsorbing material, a sufficient reproducibility in the resulting count rate was obtained. In some cases, when they are scanned by a slit aperture, the drying rings are reflected in the recorded spot activity profile.

The poly(methyl methacrylate) standards also were obtained from The Radiochemical Centre, Amersham, and their strengths at the time of delivery were, for the tritium standard, TRP 3(10 mCi/g), 1.7×10^{4} electrons/cm^{2}·sec, and for the

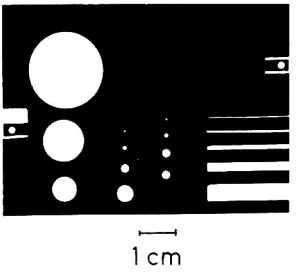


Fig. 7. Stainless steel mask of thickness 0.2 mm, intended for calibration measurements.

radiocarbon standard, CFP22 (528 μCi/g), 2.2 $\times$ 10^4 electrons/cm^2·sec. No data are available as yet for the electron energy distribution in the two cases.

In combination with the standards, a specially made mask of 0.2-mm stainless steel, containing series of holes and slits of systematically varying dimensions, was used (see Fig. 7).

Angular field of view

For a detector suspended over a chromatogram with its aperture pointing downwards, the angular field of view must be regarded as decisive both for the resulting count rate over an extended source and for the obtainable spatial resolution for a detailed activity distribution.

If it is assumed that the surface of chromatographic spots emits electrons with no preferential direction, it becomes useful to know how the probability of impinging electrons being counted, varies with the angle between their tracks and the normal to the aperture of the counter.

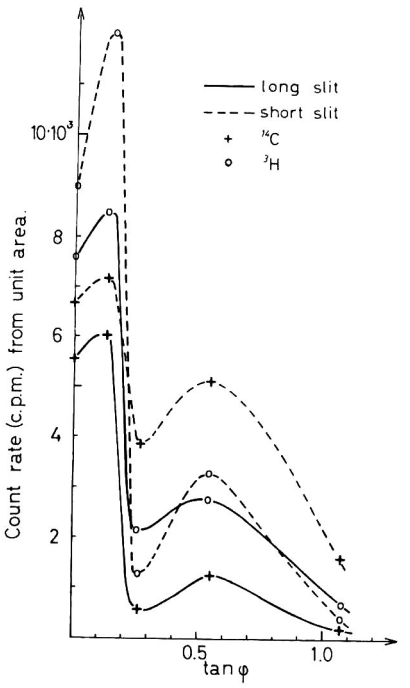
In order to investigate this point, a series of measurements were made using the previously mentioned thin metal mask in combination with the ^{3}H- and ^{14}C-standards. The mask was simply placed on top of the standards so that electrons were emitted through all the slits and holes. The slits were positioned one at a time vertically below, and parallel to, the aperture of the side slit counters and the count rates were noted. True count-rate differences divided by the corresponding differential slit areas yield the approximate angular dependence of the count rate for a unit area of isotropic electron emission. This dependence will not be corrected for the increase with the angle of the distance between the source element and the counter. This corresponds closely, however, to the normal application of the detector.

For the end-opening counters, having circular apertures, the holes in the frame were used in an analogous manner.

Two side-slit counters and two end-opening counters were tested. In both cases, one had a large and the other a small aperture.

In Fig. 8 are shown the curves obtained for the side-slit counters, with ^{3}H- and ^{14}C-electrons, respectively. The corresponding results for the two end-opening detectors are shown in Fig. 9. In both figures, the count rates are plotted against tanφ where φ is the angle between the source position and the normal to the counter aperture.

However, the curves shown are obtained with the poly(methyl methacrylate) standards, which are assumed to have an isotropic electron emission. It cannot be



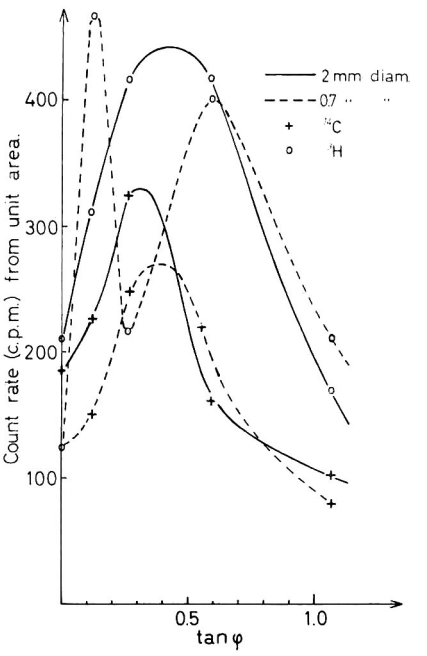


Fig. 8. Count-rate response of the side-slit detectors *vs.* the tangent of the angle between the detector normal and the direction to a line source element parallel to the slit. No corrections are made for the varying distance between the detector and the isotropic line source.

Fig. 9. Count-rate response of the end-opening detectors *vs.* the tangent of the angle between the detector normal and the direction to a circular zone element. No corrections are made for the varying distance between the detector aperture and the isotropic source element.

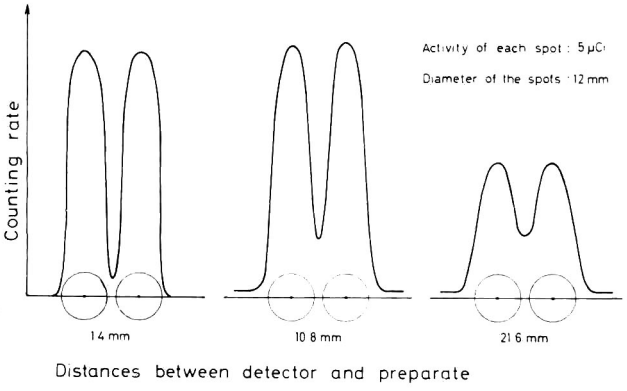


Fig. 10. The pen trace corresponding to a pair of ^{3}H-spots on Eastman Chromagram sheet (silica gel) for three different detector distances (1.4, 10.8 and 21.6 mm). On the same scale are shown the spots with their centres and drying rings. The distance between the drying rings is about 2 mm, their diametres about 12 mm and their activities 5 μCi (tritium-glucose).

taken for granted that the emission is such in the ordinary case where the activities are adsorbed on to paper or thin layers of cellulose, alumina or silica. In fact, our results seem to indicate that the electron emission is dominated by a component perpendicular to the surface of the alumina and silica preparations. This is discussed below.

Spatial resolution

As one might expect, the resolution of closely spaced spots decreases when the distance from the detector is increased. However, this decrease in resolution (and signal intensity) is much less than would be expected for spots with no angular variation in electron flux.

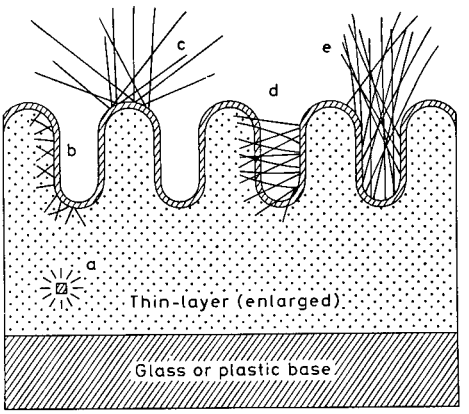


Fig. 11. Classification according to effectiveness of β-radiation from a spot of activity adsorbed on the surface of a thin-layer medium. For further description, see text.

The spatial resolutions obtained with three different counter-to-specimen distances for the same pair of ^{3}H-spots are shown in Fig. 10 for the large side-slit counter. It is assumed that the high resolution is due mainly to an angular variation in the electron flux, which is what one might expect for low-energy electrons emitted by an active substance covering a highly porous surface of a material with high stopping power.

We think, in fact, that the microgeometry of the electron radiation from activity spots in silica and alumina thin layers is better described in the form shown in Fig. 11. Possibly, the various spot materials tend to concentrate in the surface of the thin layers while the chromatograms are drying[7]. In any case, activity situated in the bulk of the layers (a) will in practice have a low efficiency, at least for tritium, owing to the small β-range associated with this nuclide. The β-radiation from the activity-covered grains and ducts in the upper surface may be classified as belonging to one of four categories: radiation inwards in a grain (b) will be ineffective. Radiation will be emitted from the top of grains (c) in all directions. However, a relatively small amount of active material is contained in such (c) positions. The same activity which is responsible for the radiation of type (b) will give radiation also of type (d), which will be absorbed in the neighbouring grains. Finally, active material on the

walls and bottoms of the pores between the grains will emit electrons in almost vertical directions (e) which will not be absorbed in the layer but will instead be radiated from the spots of activity. We suppose this to be the reason for a prevailing vertical β-emission, and suggest this as an explanation for the results described in Fig. 10. To some extent, the narrowness of the peaks in Fig. 10 might reflect the sharp angular dependence of the counter as shown in Fig. 8. In the case of radiocarbon, the effect should be much less pronounced. This has not been investigated. One additional reason for the observed decrease in resolution when the detector distance is increased is that one component of the noise signal is found to be proportional to this distance. This component might be due to more or less isotropic background radiation inside the vacuum chamber. A theoretical discussion of the obtainable resolution or "fidelity" of radiochromatogram scans has been presented earlier by JOHNSEN[8]. Without going into such detail, we can state that a practically obtainable resolution with our scanner is of the order of 1 mm. This is judged from measurements made with the standards and the metal mask and also from the results shown in Fig. 10, both for the side-slit counter.

Linearity of detection

For practical applications, it is important to establish the detector response to various amounts of radioactive material contained in the chromatogram spots. A linear dependence of the recorded signal upon the activity is preferred. A series of four different ^3H activities, ranging from 5 to 5000 nCi, were made and their activity

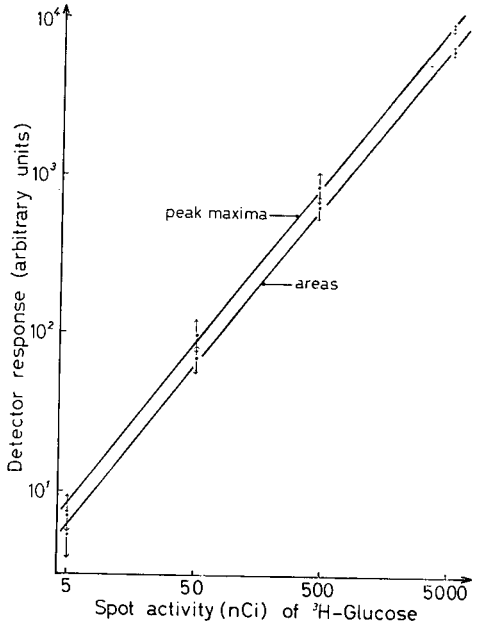


Fig. 12. Count response *versus* ^3H activity in nCi (log–log diagram). The responses are corrected for background counts. The upper curve represents count numbers in four minutes over the spot maxima. The lower curve represents areas under the spot activity profiles. (Eastman Chromagram sheet, silica gel).

profiles recorded, twice each. The results are given in Fig. 12. Two types of reading were made. In both cases, the maximum, mean and minimum values which could be read from the recordings are indicated. The count rates over each peak give the upper curve. The lower curve is drawn between corresponding values obtained as the areas under each peak. These (relative) values result from weighing the areas of recording paper after scissoring. In both cases the background signal was subtracted. Two straight lines could be drawn which always lay between the maximum and minimum values. These methods of controlling the linear response of a detector have been discussed earlier[7], where it was concluded that the comparison between areas is the preferred method, especially when evaluating spots of varying compounds and after different travel lengths in an actual chromatogram, which may cause the spot diameter to vary.

TABLE I

APPARENT COUNTING EFFICIENCIES OF FOUR THIN LAYERS WITH 5 μCi [^{3}H]GLUCOSE

The spot profiles are integrated to yield the given relative values. Correction for varying spot size has been attempted (see text).

Adsorbent type	*Relative apparent counting efficiency*	*Diameter of spot (mm)*	*Relative areas*	*Corrected relative apparent counting efficiency*
7736H, silica gel (Merck)	1	6	1	1
Whatman paper, SG81, AH81	2	9	2.3	0.9
Eastman Chromagram, K301-R2	3	12	4	0.8
7731G, silica gel (with gypsum) (Merck)	14	9	2.3	6.2

The apparent counting efficiency for a particular counter detecting tritium electrons was found to depend upon the type of adsorbent material or chromatographic paper used. This might be due to differences in activity deposition within the various media.

In Table I are shown the apparent counting efficiencies for four different adsorbent layers with a counter distance of 1.4 mm. The drying rings, being of slightly different diameters, indicate that the spot areas are different and thus that a somewhat varying amount of active material (glucose) may be deposited in the bulk of the layers, which may be of somewhat different thicknesses. The areas under the plotter curves for each of the spots have been integrated to yield the values given in Table I. It can be seen that the silica gel with gypsum from Merck gives a much higher apparent counting efficiency than the other media, and thus seems superior for low-activity measurements. In the last column are given apparent counting efficiencies corrected for varying layer thicknesses. It is assumed that a thick layer will give a small spot and little material will be absorbed at the layer surface. The corrected values have resulted from division by the respective spot areas. If surface concentration occurs during drying[7], the correction is too great and the true values will lie between the corrected and the uncorrected ones.

Background and noise

Even in the absence of radioactive material near the counter, there is still a background count rate. This is caused by the radioactivity in the counter wall materials themselves, the background radiation in the vacuum chamber, and the thermal release of electrons from the dynode strip coating. However, mean values of total noise count rates have been found as low as 1 count in 10 min with no background screening other than the walls of the vacuum container made from 2-mm stainless steel. This is an extremely low value compared with GM counters. It is certainly important to select materials free from radioactivity for the manufacture of the detector. Secondly, its very small volume compared with the sensitive volume of a GM counter is important for minimizing the influence of background radiation. More precisely, our detector has a sensitive surface layer (that of the inside of the mouthpiece) rather than a sensitive volume as in the case of the GM tube.

Total count numbers N_t and total count rates $\dot{N}_t$ must still be corrected by subtracting the corresponding background values N_b and $\dot{N}_b$ to yield the source-count and -rate, N_s and $\dot{N}_s$, respectively. However, due to the fact that both the source signal and the background are noisy in the sense that their values fluctuate due to their statistical origin, mean count rate values over some practical time interval must be established.

Assuming Poissonian statistics, the standard deviation, which may be alotted to an established mean count value as uncertainty limits, is given by:

$$\sigma_{N_s} = \sqrt{N_t + N_b} = \sqrt{N_s + 2N_b}$$

assuming no error in timing, and for count rates:

$$\sigma_{\dot{N}_s} = \sqrt{\dot{N}_t + \dot{N}_b} = \sqrt{\dot{N}_s + 2\dot{N}_b}$$

It is assumed here that the source signal can never be obtained free of its background, but the background with its standard deviation can be established by a separate measurement in the absence of the sample (source). The background count rate of the various detectors employed varied considerably. While it has been observed as low as 1 count in 10 min, it may for other detectors be sixty times greater.

The background count rate was found to consist of two components, one originating either from electrons released by field emission or due to radioactivity in the wall materials of the counter itself, or both, and the other possibly being due to thermal electrons traversing the vacuum chamber in all directions. The count level of the latter component was found to increase proportionally with the distance between the counter and the surface underneath.

During our experiments we have damaged one or two detectors by applying a high voltage in the corona range of pressure. This permanently increases the noise pulse rate.

We have also contaminated one or two detectors with ^{3}H-containing particles from the thin layers. The detectors could be washed with alcohol without being damaged, but we did not succeed in returning to the originally low background count rate. As demonstrated in the next section, a low background count rate is very

important to the achievement of a low limit of detection. Owing to the fluctuating signal and background, faint activity spots become difficult both to locate and to measure. The manner in which the above standard deviations are reflected in percentage errors in estimated peak activities has been shown earlier[8]. We shall look somewhat closer into this in connection with the performance of our detector.

Limit of detection

Provided that the activity distribution can be represented by a Gaussian distribution and that the slit width (S) of the detector is small, so as not to increase in the recording the full width at half-maximum (H) of the spot profile (in the scan direction), one can express[7] the minimum detectable activity ($A_{\min}$) as

$$A_{\min} = 1.065\, \dot{N}_b \cdot H/S \cdot \eta.$$

Here η is the overall counting efficiency (c.p.m./d.p.m.) for a detector with no slit, so that all electrons travelling upwards are collected by the counter. We shall refer to this formula without going further into the theory behind it in order to show how important a low background count rate and a high counting efficiency are when one wishes to have a low level limit of detection.

For a GM windowless gas-flow counter, BLEECKEN *et al.*[7] found η equal to 42% (c.p.m./d.p.m.) for a spot of [^{3}H]cholesterol applied to a polished plate, while the value on a Silica Gel G (Merck) thin layer was as low as 1.6% owing to sample absorption. The corresponding value for [^{14}C]cholesterol was 47.4%.

For our particular detectors, it is difficult to establish η as the aperture is fixed in each case. However, the count rates may be compared for the various detectors over the poly(methyl methacrylate) standards. While it became important for BLEECKEN *et al.*[7] as well as for SCHULZE AND WENZEL[9] to decrease the distance between the chromatogram and the GM detector as much as possible, we have seen that this is no strong requirement in our case. We have, on the other hand, observed an angular dependence in the counting efficiency.

For our various detectors, relative values of count rates, corrected for background over the ^{3}H- and the ^{14}C-standard sheets, are given in Table II. The values are normalized by setting both efficiencies arbitrarily to unity for the large side-slit counter.

It can be seen from Table II that the various detectors have different counting efficiencies for electrons entering the apertures. It is difficult to establish a true

TABLE II

RELATIVE COUNT RATES OF THE VARIOUS DETECTORS

Detector type	*Aperture (mm)*	*^{3}H-electrons (TPR3 standard)*	*^{14}C-electrons (CFP22 standard)*
Side-slit counter, large	1 × 15	1	1
Side-slit counter, small	1 × 5	0.34	2.4
End-opening counter, large	Diam. 2.0	0.02	0.08
End-opening counter, small	Diam. 0.7	0.02	0.05

counting efficiency, η, by use of activities in thin-layer media because one does not know the proportion of the electrons from the disintegrations that actually reach the detector. By use of our standards with calibrated fluxes, the sample absorption is no longer a problem but difficulties still remain because of the angular dependence of detector response. However, for geometries resembling those of common scanning, we seem to get an approximate value for η of $(40 \pm 20)\%$ for tritium (for the large side-slit counter). It is known that η decreases as the electron energy increases for this type of detector. According to FRANK, it is 60% for 450 eV electrons (*cf.* ref. 10).

The η value of 42% (c.p.m./d.p.m.) found by BLEECKEN *et al.*[7] employing a GM counter is substantially higher as they count 84% of the electrons entering the upper 2π solid angle covered by their detector (with no slit aperture).

However, the low level limit of detection is further proportional to the background count rate. Our types of counter have a very low background when they are not damaged or contaminated. Because of this, they may still compare favourably with the GM gas-flow windowless tubes. In our case, a tritium activity of 5nCi could be detected with a contaminated detector. This is the same limit as found by BLEECKEN *et al.*[7] for their GM counter. However, with an undamaged detector with a noise count rate as low as about 1 count in 1 minute, the limit would be even smaller in our case.

For the choice between counters, a criterion put forward by LOEVINGER AND BERMAN[11] may be applied: If E is the sensitivity or counting efficiency (c.p.m./d.p.m.) of the counter, the counter with the largest E will always be the best provided that it also has the largest $E^2/\dot{N}_b$. If that with the largest E has the smallest $E^2/\dot{N}_b$, it will be advantageous for (sufficiently) high spot activities and disadvantageous for (sufficiently) weak activities. This criterion, developed for the counting of radioactive samples, is also thought to be valid for detectors used in radiochromatogram scanning.

Finally, when double-lable experiments are performed, *e.g.* with ^3H and ^{14}C, one may scan the chromatogram twice, firstly recording both radionuclide activities and secondly detecting only the ^{14}C activities. This is achieved by means of a thin sheet of plastic covering the chromatogram. This effectively shields out the ^3H electrons owing to their low range in solid material, while the attenuation of the ^{14}C electrons will be negligible. The two resulting activity profiles or activity charts may afterwards be compared and the ^3H- and ^{14}C-activity spots located. This technique has been described by WENZEL and co-workers[12,13].

Two-dimensional scanning

As mentioned in the introduction, we have not yet combined two-dimensional scanning with a useful readout or printing system. Some methods which might be adopted in the present system were mentioned. A manually produced two-dimensional activity chart is presented here as an example of what may be obtained with the present scanner–detector assembly.

The chromatogram shown is made from an ethanolic extract of *Papaver somniferum*. The solvents water-saturated phenol–water (100:39, w/w) and butanol–propionic acid–water (8:18:12) have been applied successively on Whatman paper No. 1 in the y- and the x-(scan-) directions, respectively. Out of an area of 57 × 45 cm, we have selected a smaller one, 19 × 16 cm, which could be placed on the chromatogram support table and scanned. The labelling is with $^{14}CO_2$ as described elsewhere[14].

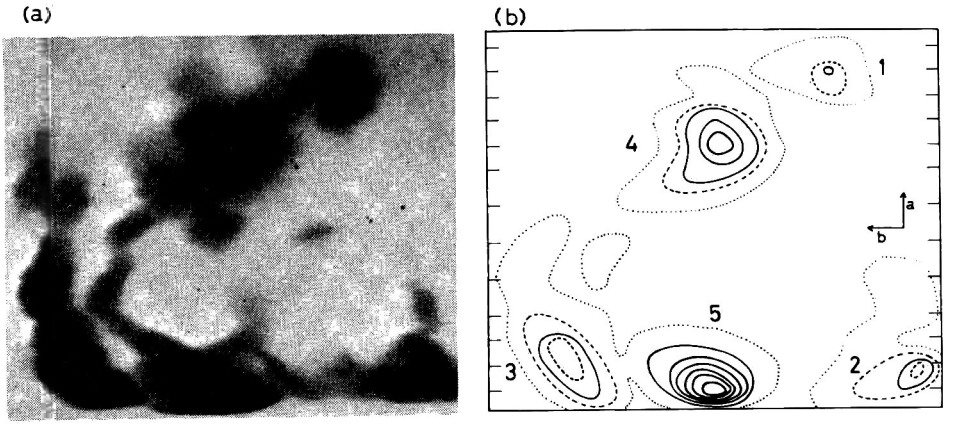


Fig. 13. Part (a) shows an autoradiogram of a two-dimensional radiochromatogram of an ethanolic extract of *Papaver somniferum*. The chromatography was performed firstly in the a- and secondly in the b-direction. Part (b) of the figure shows an iso-activity chart constructed on the basis of thirteen parallel scans, each of which has been indicated in the margin. Iso-activity contours have been drawn at intervals of 30 c.p.m. (———) and 15 c.p.m. (— — — —). An additional contour (· · · · ·) is drawn around each spot where the activity significantly increases above the background count rate. The various spots are: 1, aspartic acid (0.028); 2, meconic acid (0.031); 3, malic acid (0.052); 4, glutamic acid (0.068); and 5 citric acid (0.11). For each spot is given the integrated activity in percent of the total activity in the applied spot.

We have made 13 parallel scans of full length with a distance of approximately 1 cm between each. From the resulting activity profiles, one of which is shown in Fig. 6(a), the activity chart shown in Fig. 13(b) was constructed. A reproduction of the autoradiogram (Kodak Medical X-ray film, blue sensitive) is shown in Fig. 13(a) for comparison. The necessary exposure time for this was about 10 days. The total scan time for the 13 scans was 12 h. Isoactivity contours are drawn in the activity chart at intervals of 30 and 15 c.p.m. It is clear from this demonstration that useful two-dimensional scans may be produced automatically by the present equipment. Probably the computer-aided printout version is preferred.

CONCLUSIONS

The radiochromatographic detection of tritium-labelled compounds is still not successfully solved, but many methods have been proposed, see for example the review article by POCCHIARI AND ROSSI[15]. All the detection methods discussed, both one- and two-dimensional, can be applied to ^{14}C and other radionuclides with higher β-energies, but for tritium most of these methods have a very low counting efficiency. This is also true of a development made by HARIHARAN *et al.*[16], which is effective in locating ^{14}C compounds in two dimensions. This development is based mainly on two older works[17,18]. In one[17], a GM counter which is scanned in parallel tracks over the two-dimensional chromatograms is used. In the other[18], an array of GM counters is placed along the track of one-dimensional chromatograms. By use of 30 counters spaced at 1 cm intervals, an activity profile 30 cm long and with a resolution of about 1 cm can be obtained 30 times faster than when one single scanned detector is used.

The method presented in this paper might have advantages for tritium de-

tection firstly because the measurements are made *in vacuo*, thus eliminating the attenuation of the β-radiation between source and detector, and secondly (and we believe this to be the most important) because of the low background count rate of the electron multiplier detectors. However, the vacuum requirement can be somewhat cumbersome in practice.

A successful development of our principle of detection seems to give a higher resolution but a smaller sensitivity than another principle of detection which will be mentioned in conclusion. This new principle of detection has quite recently been applied in two commercially developed systems, one by Panax Equipment Ltd., Great Britain, called a spark chamber, and one by Baird Atomics Inc., U.S.A., called a β-camera. The first, relatively cheap, model provides a photograph of the spark pattern in a thin, flat, ionization chamber volume located closely above the chromatogram. The other, rather expensive unit provides various choises of photographic and electronic readouts. This version is based on the simultaneous use of 1622 individual windowless gas-flow GM counters. The β-camera has been briefly described by SNYDER[19].

A paper giving a more thorough discussion of available detection methods in radiochromatography is under preparation, and the emphasis there will be upon the localization and measurement of tritium activities.

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REFERENCES

1 S. PRYDZ, T. B. MELO, J. F. KOREN AND T. KRISTIANSEN, *V Symp. Chromatogr. Electrophoreses, Brussels, 1968*, pp. 159–164.
2 FARNSWORTH, *U.S. Pat.*, 1.969,399 (1930).
3 J. ADAMS AND B. W. MANLEY, *Electron. Eng.*, 37 (1965) 180.
4 P. S. OSCHEPKOV, B. N. SKVORCOV, B. A. OSANOV AND I. V. SIPRIKOV, *Prib. Tekh. Eksp.*, 4 (1960) 89.
5 G. W. GOODRICH AND W. C. WILEY, *Rev. Sci. Instrum.*, 33 (1962) 761.
6 B. FJELD AND S. PRYDZ, *J. Sci. Instrum.*, in press.
7 S. BLEECKEN, G. KAUFMANN AND K. KUMMER, *J. Chromatogr.*, 19 (1965) 105.
8 M. J. JOHNSEN, *J. Chromatogr.*, 20 (1965) 100.
9 P. E. SCHULZE AND M. WENZEL, *Angew. Chem.*, 74 (1962) 777.
10 J. ADAMS AND B. W. MANLEY, *Philips Tech. Rev.*, 28 (1967) 156.

11 R. LOEVINGER AND M. BERMAN, *Nucleonics*, 9 (1951) 26.
12 M. WENZEL, *Naturwissenschaften*, 52 (1965) 129.
13 M. WENZEL, I. JOEL AND W. OELKERS, *Atomlight (New England Nuclear Corp.)*, 54 (1966) 1.
14 A. NORDAL AND A. A. BENSON, *Plant Physiol.*, 44 (1969) 78.
15 F. POCCHIARI AND C. ROSSI, *J. Chromatogr.*, 5 (1961) 377.
16 P. V. HARIHARAN, G. POOLE AND H. E. JOHNS, *J. Chromatogr.*, 32 (1968) 356.
17 S. ARONOFF, *Nucleonics*, 14 (1956) 92.
18 C. W. GILBERT AND J. P. KEENE, in R. C. EXFERMANN (Editor), *Radioisotopes in Scientific Research, Proc. UNESCO Int. Conf., Paris, 1957*, Vol. I, Pergamon Press, London, New York, Paris, Los Angelos, 1957, p. 698.
19 F. L. SNYDER, *Isotop. Radiat. Technol.*, 6 (1969) 381.

CHROM. 5353

CHROMATOGRAPHIC PROPERTIES OF THE EPIMERIC ESTRIOLS

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SUMMARY

Thin-layer and gas chromatographic methods to separate the epimeric estriols are described. Gas chromatography of the acetates provided separation of all four. Thin-layer chromatography on Silica Gel G separated the *cis* from the *trans* estriols. The *trans* epimers separated only on silica gel modified with ammonium bisulfate. The acetonides separated well on both thin-layer and gas chromatography.

INTRODUCTION

Interest in the epimeric estriols was stimulated by the isolation of 16-epiestriol from monkey urine[1]. This substance was shown to be a major metabolite of both estrone and estradiol-17β in the rhesus monkey. Proof of the structure required an investigation of the chemistry and chromatographic behavior of the four epimeric estriols. The results of these experiments and the use of a new thin-layer medium are described in this paper. Methods involving both gas (GC) and thin-layer chromatography (TLC) will separate the epimers of estriol and the other common estrogens including the 2-hydroxy derivatives.

MATERIALS AND METHODS

All solvents for TLC were of analytical grade and redistilled before use. The thin-layer plates were all of Silica Gel G. These included Silica Gel G unmodified, Silica Gel G impregnated with phosphomolybdic acid[2] and Silica Gel G to which alcoholic ammonium bisulfate had been added[3].

The estrogens as listed below were obtained as follows: Estrone, estradiol-17β and estriol (16α,17β) and 16-epiestriol (16β,17β) were purchased from Sigma Chemical Company, St. Louis, Mo. 3,16α,17α-Trihydroxy-1,3,5,(10)-estratriene (17-epiestriol) (16α,17α) and 3,16β,17α-trihydroxy-1,3,5,(10)-estratriene (16,17-diepiestriol) (16β,17α) were generous gifts from Dr. MAX N. HUFFMAN, Creighton University, Omaha, Nebr. All of these materials on both TLC and GC showed little or no impurity and

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were used without further purification. Solutions of these steroids were made in acetone solution to give 1 μg/μl. Spotting from these solutions for TLC was performed with 10-μl Unimetric microsyringes. The chromatograms were developed in the designated solutions in the ascending manner until the solvent had reached 1 cm from the top edge of the 20 × 20 cm plates. The solvent systems used for TLC are: (A) 15% ethanol in benzene; (B) 20% ethanol in benzene; (C) 10% ethanol in benzene; (D) 2% ethanol in benzene; (E) 3% ethanol in benzene.

GC was performed with a Glowall Model 210 chromatograph which contained a detector with a 22.5 μCi radium foil. A 6 ft. × 4 mm glass coil column, containing Supelcoport (Supelco Inc., Bellefonte, Pa.) coated with 5% of OV-210 and $2\frac{1}{2}$% of OV-1 together, was used for the free estrogens. For acetylated estrogens the same column packed with Supelcoport with 5% OV-1 was required. Operating conditions were: oven and detector temperature 245°, with an argon gas flow of 60 ml/min at 30 p.s.i. for both columns.

The retention times in GC were calculated as mm from the injection peak to the peak of the curve representing the eluted compound. All relative retention times were calculated from estrone or estriol as 100.

The acetates were made by dissolving the steroid (100 μg) in 6 drops of pyridine and then adding 3 drops of acetic anhydride. After remaining for 18 h at room temperature the reaction mixture was evaporated by a stream of nitrogen while warming the containers in a water bath. The acetonides were made by a modification of the method of HUFFMAN AND LOTT[4]. To the steroid (100 μg) in 1 ml of acetone was added 0.5 ml of acetone saturated with dry HCl. (The acetone was saturated with HCl while cooled in an ice bath.) After 15 min at room temperature, the HCl was neutralized with 5% sodium bicarbonate. The acetonide was extracted with ether after removal of acetone *in vacuo*. These conditions gave essentially complete reaction of the steroid with the ketone; no peak other than the acetonide was seen after GC.

RESULTS

Gas chromatography

The free *cis*-estriols are not amenable to GC because of decomposition at the high temperatures required. The separation of these free estriols was not possible by GC. The *cis* epimers presented flattened and broad peaks in the recorder tracings while the *trans* epimers gave symmetrical peaks but did not separate. The presence of the 2-hydroxy increased the retention time of the estrogens. In contrast, the acetates of all four epimeric estriols showed sharp symmetrical peaks and separated well as indicated in Table I.

The acetonides of the epimeric *cis*-estriols had retention times shorter than that of the free estriols. The *trans* epimers did not react with the acetone. The relative retention time for estriol-16α,17α was 57, while that for the β,β-epimer was 68 compared to free E_3. Table II gives the relative retention time of the acetonides of the two epimers with two different packings in the GC columns in comparison with the unreacted steroids.

TABLE I

GC RELATIVE RETENTION TIMES FOR ESTROGENS

Calculated as mm from injection peak to the peak of the curve.

Estrogen	*3½% OV-210*[a] *+ 1½% OV-1*	*5% OV-1*[a]
Estrone (E_1)	100 (12 min)	
2-OH-Estrone[b]	205	
Estradiol-17β (E_2)	80	
2-OH-estradiol-17β	158	
Estriol-16α,17β (E_3)	146	
2-OH-estriol-16α,17β	290	
Estriol-16β,17α	148	
E_1-acetate		100
2-OH-E_1-acetate		194
E_2-acetate		138
2-OH-E_2-acetate		272
E_3-acetate-16α,17β		234
2-OH-E_3-acetate-16α,17β		462
E_3-acetate-16β,17β		277
E_3-acetate-16α,17α		286
E_3-acetate-16β,17α		270

[a] For description of column packings see text.
[b] 2-OH = 2-hydroxy.

TABLE II

GC RELATIVE RETENTION TIMES OF ESTRIOL ACETONIDES

Free estriol = 100.

Estrogen acetonide	*3.5% OV-210 + 1.5% OV-1*	*5% OV-1*
E_3-16α,17β (N.A.)[a]	100	100
E_3-16β,17β	90	68
E_3-16α,17α	74	57
E_3-16β,17α (N.A.)	104	96

[a] N.A. = no acetonide was formed.

TABLE III

TLC R_F VALUES OF ESTROGENS

Estrogen	*System A, plain plate*	*System B, H_2SO_4 plate*[a]	*System A, PMA plate*[b]
E_1	0.72	0.56	0.61
2-OH-E_1	0.54	0.44	0.43
E_2	0.60	0.46	0.55
2-OH-E_2	0.44	0.40	0.37
E_3-16α,17β	0.35	0.09	0.21
2-OH-E_3-16α,17β	0.26	0.26	0.12
E_3-16α,17α	0.47	0.44	0.19
E_3-16β,17β	0.47	0.18	0.19
E_3-16β,17α	0.35	0.09	0.23

[a] Silica gel with ammonium bisulfate.
[b] Silica gel with phosphomolybdic acid.

Thin-layer chromatography

TLC methods have provided a wide variety of possibilities for separation of estrogens. None of the conventional systems[5,6] so far studied will separate the four epimeric estriols individually. The *cis* can be separated from the *trans* epimers in conventional systems. The separations with varying solvent and plate conditions are shown in Table III. Of the three different types of media available only the modified silica gel was capable of separating the epimeric estriols. The two *trans* epimers could be separated on plain silica gel from the two *cis* epimers. On the PMA modified silica gel the *cis* isomers traveled slower than the *trans* epimers. The two *cis* epimers

TABLE IV

TLC R_F VALUES OF ESTRIOL ACETONIDES

Estrogen acetonide	*System C, plain plate*	*System A, H_2SO_4 plate*	*System C, PMA plate*
E_3-16α,17β (NA)[a]	0.06	0.10	0.05
E_3-16α,17α	0.41	0.41	0.38
E_3-16β,17β	0.41	0.24	0.38
E_3-16β,17α (NA)	0.06	0.10	0.05

[a] NA = no acetonide was formed.

showed a very wide separation in the silica gel to which NH_4HSO_4 had been added. The two *trans* epimers were not separated although they were separated from the *cis* epimers. The epimeric estriols also have mobilities close to that of 2-hydroxy-estrone and 2-hydroxy-estradiol-17β, in unmodified silica gel. As shown in Table III by modification of the silica gel separation of these compounds is more easily achieved.

The separation of the acetonides of the epimeric estriols followed the same pattern as the free epimers. Separation of the two *cis*-acetonides was achieved in the silica gel modified with NH_4HSO_4 as shown in Table IV. Table V indicates that estrogen acetates can be separated by TLC on silica gel. There is some overlapping of mobilities. Of particular interest is that after acetylation the two *cis* epimers no longer separated on the modified silica gel.

TABLE V

TLC R_F VALUES OF ESTROGEN ACETATES

Estrogen acetate	*System D, plain plate*	*System E, H_2SO_4 plate*	*System E, PMA plate*
E_1-Ac[a]	0.53	0.46	0.37
2-OH-E_1-diAc	0.27		
E_2-diAc	0.41	0.52	0.49
2-OH-E_2-triAc	0.38		
E_3-16α,17β-triAc	0.38	0.52	0.35
2-OH-E_3-tetraAc	0.28		
E_3-16β,17β-triAc	0.47	0.41	0.28
E_3-16α,17α-triAc	0.47	0.41	0.28
E_3-16β,17α-triAc	0.54	0.52	0.35

[a] Ac = acetate.

DISCUSSION

The literature lists many conditions and solvent systems for TLC separation of steroids. LISBOA[6] has compiled a list of estrogens and solvent systems which will separate them. Few, if any of the conventional systems will separate all of the estrogens in a single operation.

The use of modified adsorbents such as the silica gel containing bisulfate or phosphomolybdic acid may provide a means for more specific separations in situations such as existing in the problem of separation of the epimeric estriols. GC along with TLC provides a useful tool for separation and quantitation of many steroids. The separation of the estriol acetates was readily achieved.

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REFERENCES

1 M. BRECKWOLDT, G. L. FLICKINGER, T. MURAWEC AND J. C. TOUCHSTONE, unpublished results.
2 J. C. TOUCHSTONE, A. K. BALIN, T. MURAWEC AND M. KASPAROW, *J. Chromatogr. Sci.*, 8 (1970) 443.
3 J. C. TOUCHSTONE, T. MURAWEC, M. KASPAROW AND K. P. BALIN, *Pittsburgh Conf. Anal. Chem. Appl. Spectrosc., Cleveland, Ohio, March 1, 1971.*
4 M. N. HUFFMAN AND M. H. LOTT, *J. Biol. Chem.*, 215 (1955) 627.
5 R. NEHER, in E. STAHL (Editor), *Thin-Layer Chromatography*, Springer, New York, 1969, p. 311.
6 B. P. LISBOA, *Acta Endocrinol.*, 43 (1963) 47.

CHROM. 5350

STUDIES ON QUANTITATIVE *IN SITU* FLUOROMETRY OF LYSERGIC ACID DIETHYLAMIDE (LSD) ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

The quantitative *in situ* fluorometry of LSD on thin-layer chromatograms was investigated. Reproducibilities for the range 0.1–2.0 μg of LSD on the same and different chromatograms were examined in both direct and internal standard methods. The linear relationship between fluorescence emission intensity and amount of LSD was found in both methods.

This technique has been applied to the determination of unchanged LSD in the photodecomposition process. It was noted that LSD was easily decomposed by ultraviolet irradiation.

INTRODUCTION

Lysergic acid amide, isolysergic acid amide and clavine alkaloids in morning glory seeds have been determined by densitometry after treatment with Van Urk reagent on thin-layer chromatograms[1]. It is known, however, that lysergic acid diethylamide (LSD) on a chromatogram is detected by fluorescence with much better sensitivity than by coloration with the reagent[2]. Recently, various instruments and methods for *in situ* fluorometric scanning of thin-layer chromatograms have been developed[3–8].

This paper describes the quantitative *in situ* fluorometry of LSD on thin-layer chromatograms, using an automatic scanner with a digital recorder, and the reproducibilities of both direct and internal standard methods are discussed. In addition, this technique has been applied to determination of unchanged LSD in the photodecomposition process.

EXPERIMENTAL

Materials

LSD was synthesized from *d*-lysergic acid (Sigma) by GARBRECHT's method[9]. Quinine was recrystallized from benzene.

Apparatus

A Hitachi MPF-2 type Autorecording Spectrophotofluorometer equipped with a scanning attachment for thin-layer chromatograms, a J201 integrator and a J301 digital recorder (Hitachi Ltd.) was used with the following settings: sensitivity, 6; slit width for excitation and emission, 10 and 10 mμ, respectively; slit for thin-layer chromatograms, 6 × 2 mm. The fluorescence emission profiles were obtained on a recorder, and at the same time the fluorescence emission intensities were printed out as numerals on a digital recorder. The scanning direction of the chromatograms was either parallel or perpendicular to the chromatographic flow.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on 250-μ layers of Silica Gel G. Solvent systems used for development were: (A) methanol–chloroform–*n*-hexane (1:4:2), (B) methanol–chloroform (1:4), and (C) acetone–chloroform (4:1). After development, the plates were dried for 5 min in a stream of warm air.

Direct method

5 μl of chloroform solution containing 2.0 μg of LSD were spotted at a starting point on a chromatographic plate and four more spots were prepared on the same plate, followed by development with three solvent systems. The fluorescence emission intensity of LSD on the chromatogram was measured and the relative standard deviation on the same chromatogram was calculated. The chromatography was repeated five times on different plates and the relative standard deviations on different chromatograms were calculated. In the same way, the relative standard deviations on both the same and different plates were obtained from solutions containing 1.5, 1.0, 0.5, 0.2 and 0.1 μg of LSD, respectively, and the relationship between emission intensity and the amount of LSD was examined.

Internal standard method

Quinine was used as an internal standard. 5 μl of ethanol solution containing 2.0 μg of LSD and 0.5 μg of quinine were spotted at a starting point on a chromatographic plate, four more spots were prepared on the same chromatographic plate, and the development was carried out in the solvent systems (A) and (B). The ratio of the fluorescence emission intensity of LSD to that of quinine was obtained. The relative standard deviation of this ratio on the same plate was calculated. This chromatography was repeated five times on different plates and relative standard deviations on different chromatograms were calculated. The same procedure was repeated on solutions containing 1.5, 1.0, 0.5, 0.2 and 0.1 μg of LSD, and 0.5 μg of quinine, respectively. The relationship between the above ratio and the amount of LSD was examined.

UV irradiation for photodecomposition of LSD

2 μg of LSD spotted on a thin layer were irradiated with UV light from a National GL-10 lamp (2537 Å, distance 10 cm) for 1, 5, 15, 30 and 60 min. After irradiation, three or four standards containing 0.2–2.0 μg of LSD were spotted on the same plate and TLC was carried out using the solvent systems (A), (B) and (C). Amounts of unchanged LSD on the chromatograms were determined by the direct

method. As a control experiment, 2 μg of LSD spotted on thin layers were left to stand in darkness for 60 min or 17 h, followed by chromatography and determination of unchanged LSD as above.

RESULTS AND DISCUSSION

Direct method

The excitation and emission wavelength for the direct method were selected to give maximum emission intensity of the spot of LSD on the chromatogram. They were 330 and 410 mμ, respectively, as shown in Fig. 1. The reproducibilities for the range 0.1–2.0 μg of LSD in the direct method are shown in Table I.

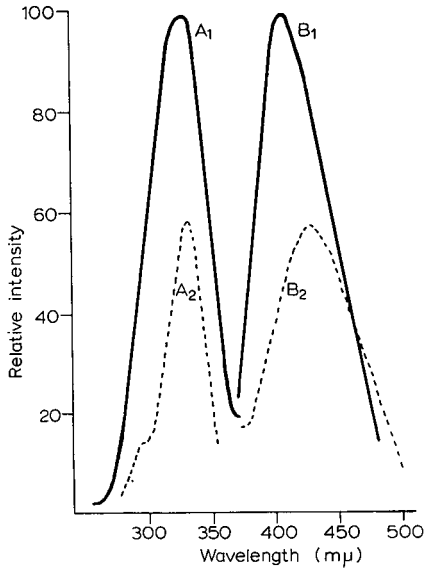

Fig. 1. Fluorescence excitation and emission spectra for LSD and quinine on a thin-layer chromatogram. Mixture containing 2.0 μg of LSD and 0.5 μg of quinine were spotted on the thin-layer plate, followed by development in the solvent system (A), MeOH–$CHCl_3$–*n*-hexane (1:4:2). (A_1) Fluorescence excitation spectrum for LSD by setting emission at 410 mμ; (B_1) fluorescence emission spectrum for LSD by setting excitation at 330 mμ; (A_2) fluorescence excitation spectrum for quinine by setting emission at 436 mμ; (B_2) fluorescence emission spectrum for quinine by setting excitation at 336 mμ.

Relative standard deviations obtained by scanning in the direction parallel to the chromatographic flow were 0.7–6.3% on the same chromatogram and 3.3–10.8% on different chromatograms. For the case of scanning in the direction perpendicular to the chromatographic flow, higher deviations were observed than those shown in Table I. Accordingly, in this experiment, scanning of the chromatograms was performed in the direction parallel to the chromatographic flow. The relationship between emission intensity and the amount of LSD was linear over the range 0.2–2.0 μg in each solvent system (Fig. 2).

TABLE I

REPRODUCIBILITIES IN THE DIRECT METHOD

Solvent system	*LSD (μg)*	*Standard deviation (%)*			
		Parallel to chromatographic flow		*Perpendicular to chromatographic flow*	
		Same chromatogram	*Different chromatograms*	*Same chromatogram*	*Different chromatogram*
(A)	2.0	0.7–3.4	5.6	0.3– 3.8	5.6
	1.5	2.9–5.4	10.1	2.8– 5.6	10.8
	1.0	2.3–2.7	9.7	1.8–11.3	14.5
	0.5	3.2–4.5	6.6	2.5–12.1	4.7
	0.2	1.2–3.3	6.4	4.6– 8.7	9.9
	0.1	3.7–6.3	8.9	5.3– 8.0	3.8
(B)	2.0	1.6–4.0	5.2	0.8–11.2	5.9
	1.5	3.6–4.5	9.3	2.8– 4.1	14.6
	1.0	2.1–4.7	10.8	5.5– 8.6	16.2
	0.5	1.0–4.0	8.0	1.0–11.2	15.8
	0.2	3.3–5.9	4.3	6.1– 8.2	21.3
	0.1	2.8–5.1	5.5	2.1– 7.4	11.5
(C)	2.0	0.9–3.2	3.3	0.3–10.7	12.2
	1.5	3.4–4.4	9.4	3.4– 9.7	11.0
	1.0	1.6–4.9	6.1	3.1– 7.0	8.1
	0.5	3.1–4.5	9.0	6.9–13.7	5.5
	0.2	2.0–4.4	6.5	0.9–15.0	10.0
	0.1	3.9–5.6	5.9	5.1–14.9	8.5

Internal standard method

The excitation and emission wavelength described for the direct method were used in the internal standard method, although the maximum emission intensity of quinine which was used as an internal standard compound was obtained at 436 mμ by excitation at 336 mμ, as shown in Fig. 1. The intensity of quinine decreased to about 75% of the maximum intensity by excitation at 330 mμ and emission at 410

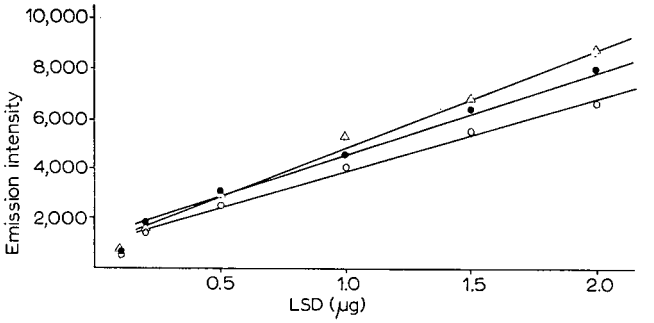

Fig. 2. Relationship between fluorescence emission intensity and amount of LSD. ●—●, solvent system (A), MeOH–$CHCl_3$–*n*-hexane (1:4:2); ○—○, solvent system (B), MeOH–$CHCl_3$ (1:4); △—△, solvent system (C), acetone–$CHCl_3$ (4:1).

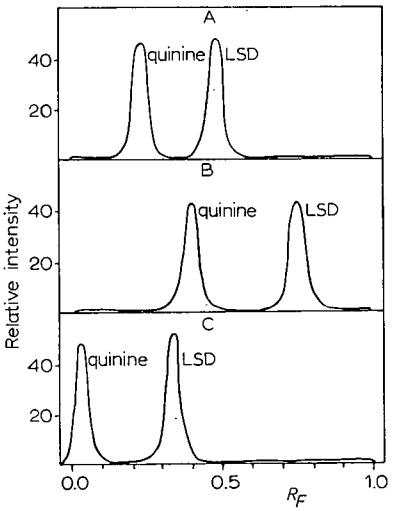

Fig. 3. Fluorescence emission profiles of a mixture containing 1.0 μg of LSD and 0.5 μg of quinine. (A), Solvent system (A); (B), solvent system (B); (C), solvent system (C).

mμ, and 0.5 μg of quinine on a chromatogram gave an intensity about half that of 2.0 μg of LSD.

LSD and quinine were separated on the chromatograms in the solvent systems (A), (B) and (C) as shown in Fig. 3. However, systems (A) and (B) were used since quinine stayed at the starting point of the chromatogram in system (C).

Relative standard deviations for the range 0.1–2.0 μg of LSD were 0.2–6.2% on the same chromatogram and 1.6–9.3% on different chromatograms, as shown in Table II.

TABLE II

REPRODUCIBILITIES IN THE INTERNAL STANDARD METHOD

Solvent system	*LSD (μg)*	*Standard deviation (%)*	
		Same chromatogram	*Different chromatograms*
(A)	2.0	0.2–5.8	7.9
	1.5	1.9–4.8	1.7
	1.0	3.4–5.2	4.2
	0.5	0.8–5.6	9.3
	0.2	2.7–6.2	6.1
	0.1	3.6–4.2	5.5
(B)	2.0	1.7–4.6	2.0
	1.5	3.4–5.6	1.6
	1.0	2.7–3.1	5.9
	0.5	4.3–4.5	5.2
	0.2	1.7–5.2	6.6
	0.1	2.5–5.2	3.6

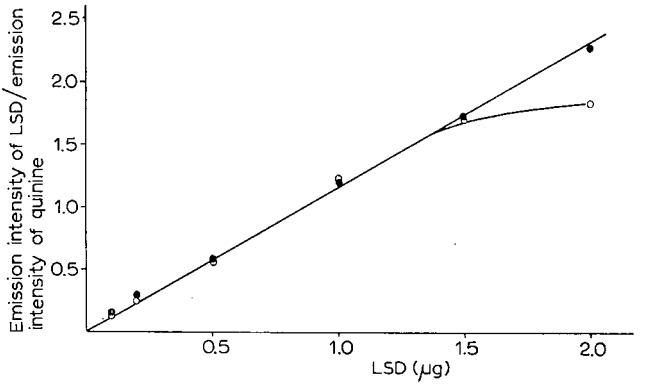

Fig. 4. Relationship between the ratio of the fluorescence emission intensity of LSD to that of quinine and the amount of LSD. ●—●, solvent system (A); ○—○, solvent system (B).

The relationship between the ratio of the emission intensity of LSD to that of quinine and the amount of LSD was linear over the range 0 – 1.5 μg (Fig. 4).

The reproducibilities of scanning on the same plate were found to be less than 6% in both the direct and internal standard methods, whilst those on different plates were less than 9% in the internal standard methods and less than 11% in the direct method.

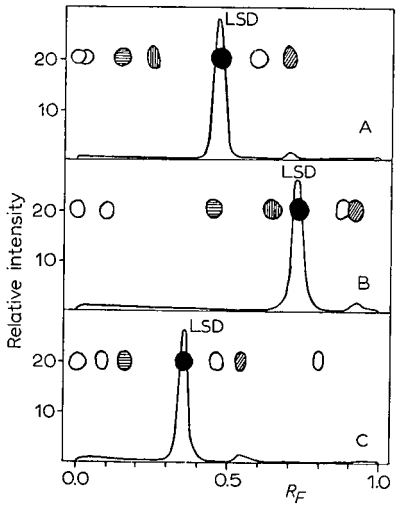

Fig. 5. Fluorescence emission profiles of UV-irradiated LSD. (A) Solvent system (A); (B) solvent system (B); (C) solvent system (C). 2 μg of LSD spotted on a thin-layer plate were irradiated with UV light (2537 Å) for 15 min. After development, spots were detected by observation under UV light at 3650 Å and then by coloration with *p*-dimethylaminobenzaldehyde reagent. ●, Detected by blue fluorescence under UV light and blue color with the reagent; ○, detected by blue fluorescence under UV light and orange color with the reagent; ▨, detected by yellow fluorescence under UV light and blue color with the reagent; ◍, detected by UV light but not by the reagent; ◍, detected by the reagent but not by UV light.

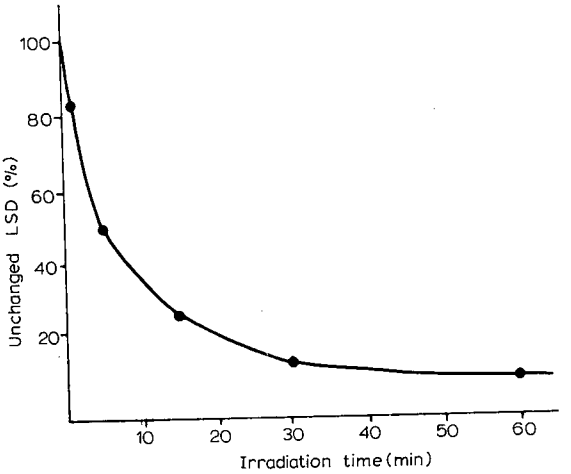

Fig. 6. Photodecomposition curve of LSD.

Photodecomposition of LSD on thin layers

The fluorescence emission profiles of chromatograms of LSD irradiated for 15 min are shown in Fig. 5. Five additional spots were detected by observation under UV light. The amounts of unchanged LSD at each irradiation time were determined by the direct method, and the photodecomposition curve is shown in Fig. 6.

The amount of 2 μg of LSD initially spotted on a thin layer was decreased to 0.2 μg (10%) by irradiation for 60 min, whilst in the control experiment, LSD in darkness was unchanged after standing for 60 min but decreased to 1.8 μg (90%) after 17 h. Structures of the products of the photodecomposition of LSD are now under investigation.

It is considered that LSD in samples containing some impurities or in biological materials can be determined practically without purification by either the direct or the internal standard method described in the present work.

REFERENCES

1 K. GENEST, *J. Chromatogr.*, 19 (1965) 531.
2 T. NIWAGUCHI AND T. INOUE, *Kagaku Keisatsu Kenkyusho Hokoku*, 22 (1969) 126.
3 R. KLAUS, *J. Chromatogr.*, 16 (1964) 311.
4 E. SAWICKI, T. W. STANLEY AND W. C. ELBERT, *J. Chromatogr.*, 20 (1965) 348.
5 D. JÄNCHEN AND G. PATAKI, *J. Chromatogr.*, 33 (1968) 391.
6 R. K. IBRAHIM, *J. Chromatogr.*, 42 (1969) 544.
7 H. ZÜRCHER, G. PATAKI, J. BORKO AND R. W. FREI, *J. Chromatogr.*, 43 (1969) 457.
8 B. C. MADSEN AND H. W. LATZ, *J. Chromatogr.*, 50 (1970) 288.
9 W. L. GARBRECHT, *J. Org. Chem.*, 24 (1959) 368.

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FLAVONES AS FLUOROGENIC SPRAY REAGENTS FOR ORGANOTHIOPHOSPHORUS PESTICIDES ON SILICA GEL LAYERS

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SUMMARY

A method is discussed for the detection and quantitative determination of organothiophosphorus pesticides by *in situ* fluorometry after separation on silica gel layers. Yellowish-green fluorescent spots are obtained when the plate is sprayed with a 3-hydroxyflavone, such as robinetin, after bromination. An attempt is made to explain this phenomenon. Linear calibration curves up to 4 μg per spot of the pesticide have been obtained and a relative standard deviation of approximately 4% can be expected at the 1.0-μg level. Visual and instrumental detection limits are around 0.04 μg per spot for certain pesticides.

INTRODUCTION

The use of flavones as fluorogenic spray reagents is relatively new. ČERNÝ *et al.*[1] used alumina layers impregnated with morin for the detection of steroids. Substances containing α,β-unsaturated keto groups formed dark spots on a yellow fluorescent background under UV light. Other compounds were visible as bright yellow spots on a darker background. In all cases 100 μg or more of the steroids had been spotted.

SCHELLENBERG[2] detected N-protected amino acids and peptide derivatives on silica gel thin-layer chromatograms after spraying the layer with a 0.05% solution of morin and heating at 100° for 2 min. Yellow fluorescent or dark absorption spots were observed under UV light. The limit of detection for most compounds was around 2 μg per spot. Morin has also been employed for the detection of phenols and nitrophenols[3].

Recently it was found[4,5] that quantities as low as 0.02 μg of some organic pesticides could be detected on cellulose layers sprayed with 3-hydroxyflavones such as flavonol, fisetin and robinetin. Yellow fluorescent spots were observed on a relatively non-fluorescent background.

In this study, the use of flavones as spray reagents on silica gel layers is investigated, since most of the thin-layer chromatographic (TLC) methods that have been reported for organophosphorus pesticides make use of this type of adsorbent.

EXPERIMENTAL

Chemicals and apparatus

The flavones fisetin, kaempferol, quercetin, morin, and rutin were purchased from Fluka A.G., Chemische Fabrik, Buchs S.G., Switzerland; chrysin, apigenin and acacetin from Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.; robinetin-aglucone from Koch-Light Laboratories, Colnbrook, Bucks., Great Britain; and flavonol from Eastman Organic Chemicals, Distillation Product Industries, Rochester, N.Y., U.S.A. Unless otherwise stated, 0.05% solutions of the flavones were prepared in ethanol.

The pesticides were supplied as analytical standards by several manufacturers as listed by KENAGA AND ALLISON[6]. Stock solutions of the pesticides (spotted by means of 1-μl Microcaps) were prepared 1000 p.p.m. in *n*-hexane from which dilution series were made. All the solvents were redistilled.

Fluorescence spectra were measured with the Aminco-Bowman Spectrophotofluorometer with TLC attachment. Quantitative work was carried out with the Zeiss Chromatogram-Spectrophotometer.

Chromatography and detection

The chromatoplates (20 × 20 cm) were coated 250 μ thick with a mixture of 30 g of Silica Gel N (Macherey, Nagel and Co., Duren, G.F.R.) in 80 ml of water by means of a Desaga TLC applicator. After drying in air the plates were placed in an oven at 105° for 30 min before use. For chromatographic separation the pesticides were spotted 2 cm from the bottom of the plates and developed 10 cm in *n*-hexane–acetone (5:1)[7].

After removal from the chromatographic chamber the plate was dried for 5 min at 105°. While the plate was still hot it was brominated for 10 sec in a chromatographic tank containing a 10% solution of bromine in carbon tetrachloride; after a few minutes of cooling it was sprayed in excess with the particular flavone. The plate was then activated in the oven for another 5 min to produce the fluorescence.

In situ qualitative and quantitative studies

The procedure for recording the fluorescence spectra with the Aminco-Bowman spectrophotofluorometer has already been described[8].

To investigate the fading behaviour with the flavone robinetin, a 1-μg sample of Trithion was spotted, eluted, brominated, and sprayed, and the disappearance of the fluorescence with time was monitored instrumentally. For reproducibility studies, nine spots were developed simultaneously on one plate and scanned in the direction of elution. In all studies the M-365 excitation filter and the emission monochromator set at 505 nm were used for optimum conditions with the Zeiss Chromatogram-Spectrophotometer. For quantitative work a 0.1% solution of robinetin in ethanol was employed.

RESULTS AND DISCUSSION

The flavones used in this study are listed in Table I. In previous work[4,5], only

TABLE I

EVALUATION OF FLAVONES ON SILICA GEL

Common name	*Chemical name*	*Fluorescence of background*
Flavonol	3-hydroxyflavone	bluish-green
Fisetin	3,3′,4′,7-tetrahydroxyflavone	light yellow
Robinetin	3,3′,4′,5′,7-pentahydroxyflavone	dark yellow
Kaempferol	3,4′,5,7-tetrahydroxyflavone	light yellow
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	slightly yellow
Morin	2′,3,4′,5,7-pentahydroxyflavone	yellowish-green
Chrysin	5,7-dihydroxyflavone	none
Apigenin	4′,5,7-trihydroxyflavone	none
Acacetin	5,7-dihydroxy-4′-methoxyflavone	none
Rutin	quercetin-3-rutinoside	light yellow

3-hydroxyflavones showed a slight yellow fluorescence on cellulose layers. However, on silica gel, which is a much more polar medium, both 3-hydroxy- and 3,5-dihydroxyflavones were fluorescent, whereas, as expected, the 5-hydroxyflavones were not. Heating in the oven for 5 min at 105° resulted in fading of the background fluorescence with the 3-hydroxyflavones in the order flavonol < fisetin < robinetin. Without heating the fading would take several hours. No change was observed for the other flavones.

Direct spraying with robinetin of silica gel plates spotted with the pesticides DDT, Trithion, Proban, Malathion, prometryne, CIPC, Sevin, and Baygon resulted in yellow fluorescent spots against a relatively quenched background after heating

TABLE II

DETECTION LIMITS OF ORGANOTHIOPHOSPHORUS PESTICIDES

Pesticide	*No. of sulphur atoms*[a]	*Visual limit of detection (μg)*
Trithion	3	0.04
Guthion	2	0.06
Parathion	1	0.1
Proban	1	0.1

[a] The influence of type and number of sulphur atoms on the fluorescence has been discussed (see ref. 8).

in the oven for 5 min at 105°. However, the method was not sensitive (~ 5 μg per spot). Considerable improvement in the detection limit was noticed with organothiophosphorus pesticides if the plate was brominated before spraying with the flavone (see Table II). As in previously discussed work[4], the fluorescence phenomenon in this method was specific to 3-hydroxyflavones with the 5-position unsubstituted. Robinetin was preferred over fisetin and flavonol, due to its lower background fluorescence.

The fluorescence spectral data for the three 3-hydroxyflavones on silica gel are shown in Table III. The spectra obtained for both Guthion and Trithion sprayed with robinetin after bromination are identical, suggesting that the fluorescence is

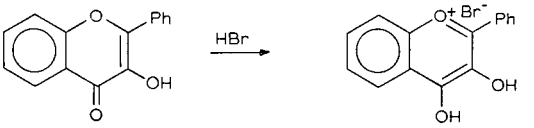
TABLE III

FLUORESCENCE SPECTRAL DATA ON SILICA GEL LAYERS

Fluorescent spot	*Wavelength (nm)*	
	Excitation	*Emission*
Flavonol	410	480
Fisetin	425	500
Robinetin	430	510
Robinetin + Guthion + Br_2	370	505
Robinetin + Trithion + Br_2	370	505
Robinetin + HBr	370	505

independent of the pesticide. Furthermore, these spectra are markedly different from those of the flavone robinetin. It is believed that upon bromination of the organothiophosphorus pesticides, hydrobromic acid is formed and eventually causes the fluorescence. The spectrum of robinetin sprayed on HBr supports this.

Fluorescence emission is caused by a change of the electronic arrangement in the ring formed through 3-hydroxyl and 4-carbonyl[9]. The effect of HBr on robinetin or any other 3-hydroxyflavone is assumed to be a protonation of the carbonyl oxygen to form a highly fluorescent species of the type shown below. Such salts are known to exist and have been isolated[10]. The phenomenon that occurs upon spraying of the

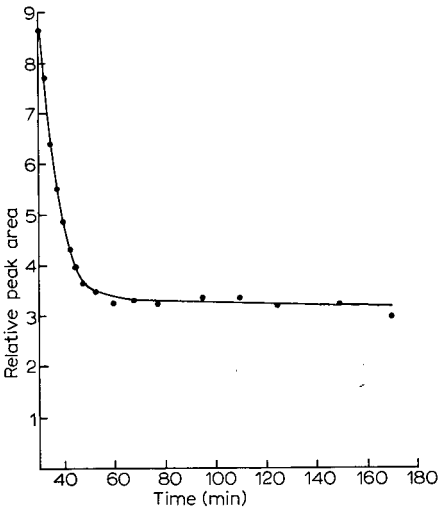


robinetin on silica gel layers (*i.e.*, the background is highly fluorescent but disappears on standing or with the influence of heat) is probably due to strong intermolecular forces, such as hydrogen bonding, which can cause quenching of the fluorescence[11]. This type of interaction is quite important on silica gel layers.

Fig. 1 illustrates the fading characteristics of a fluorescent spot obtained by spraying 1 μg of brominated Trithion with robinetin. A 62% decrease in fluorescence is observed for the first hour, followed by a zone of reasonable stability. It seems that the spot is extremely fluorescent under anhydrous conditions, but as the plate cools off and moisture is re-adsorbed the fluorescence intensity decreases until a saturation point is reached. Reproducibility studies were therefore carried out after 1-h standing in the dark. An average value of 4.7% relative standard deviation was obtained for 1-μg spots ($n = 9$) of Trithion.

Calibration plots of fluorescence *vs.* concentration for the pesticide Trithion sprayed with robinetin are shown in Fig. 2. The plots are linear up to about 4 μg per spot. The curvature above 4 μg can be attributed to concentration quenching effects which have been reported previously[8]. Below 0.1 μg the reproducibility of the method becomes rather poor and the use of standard addition techniques is recommended. Similar results were obtained with Parathion.

Various types of silica gel, such as Silica Gel H, Silica Gel HR, Silica Gel G, and Silica Gel S (all Merck reagents), and also MN Silica Gel N from Macherey, Nagel



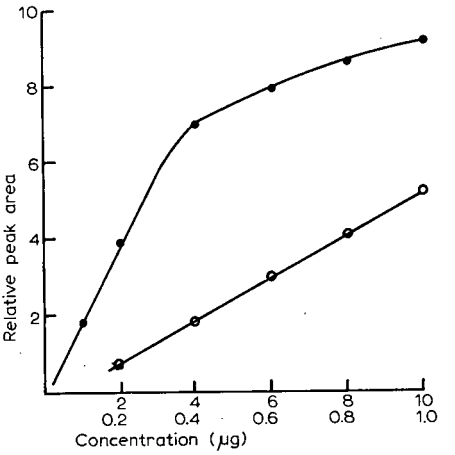


Fig. 1. Study of the fluorescent fading of a spot of brominated Trithion sprayed with robinetin.

Fig. 2. Calibration curves for Trithion. ●, 1.0–10 μg; ○, 0.2–1 μg.

and Co., have been tested. The best results were obtained with silica gel without binder. For the bromination step, the best results were obtained by brominating for 5–10 sec. Longer bromination causes an increase in background fluorescence. The influence of varying the concentration of the spray reagent was studied also. Quantitative work was feasible with 0.025, 0.05, and 0.1% solutions in ethanol (w/v), but reproducibility as well as fluorescence intensity were best at 0.1%.

CONCLUSIONS

The method described offers another approach to the determination of organothiophosphorus pesticides by *in situ* fluorometry. The possibility of being able to use flavones as spray reagents on silica gel layers is advantageous, since many separation procedures have been reported on this matrix. Even though it is slightly less sensitive and precise when compared to the SAQH–Mn procedure* described earlier[8], it is equally simple, and the reagents are readily available from commercial sources. Since linear calibration curves going through the origin are obtained below 4 μg per spot, it should be possible to apply standard addition techniques and thus increase the practical concentration range of the method. Since other parameters are the same as in the SAQH–Mn method[8], the procedure should be adaptable to the quantization of subparts per billion levels of organothiophosphorus pesticides in water samples without major clean-up and to more complex biological samples in conjunction with suitable clean-up methods. It should offer a valuable alternative to gas–liquid chromatography and serve as a check method.

* This method is similar to that published previously[12] except that the chelating agent is salicyl-2-aldehyde-2-quinolylhydrazone (SAQH).

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REFERENCES

1 V. ČERNÝ, J. JOSKA AND L. LÁBLER, *Collect. Czech. Chem. Commun.*, 26 (1961) 658.
2 P. SCHELLENBERG, *Angew. Chem., Int. Ed. Engl.*, 1 (1962) 114.
3 G. TADEMA AND P. A. BATELAAN, *J. Chromatogr.*, 33 (1968) 460.
4 V. MALLET AND R. W. FREI, *J. Chromatogr.*, 54 (1971) 257.
5 V. MALLET AND R. W. FREI, *J. Chromatogr.*, 56 (1971) 69.
6 E. E. KENAGA AND W. E. ALLISON, *Bull. Entomol. Soc. Amer.*, 15 (1969) 85.
7 J. ASKEW, J. H. RUZICKA AND B. B. WHEALS, *Analyst*, 94 (1969) 275.
8 R. W. FREI AND V. MALLET, *Environ. Anal. Chem.*, in press.
9 T. HAYASHI, K. HARA, S. KAWAI AND T. OHNO, *Chem. Pharm. Bull. (Tokyo)*, 18 (1970) 1112.
10 W. C. BLASDALE, *J. Amer. Chem. Soc.*, 67 (1945) 491.
11 B. L. VAN DUUREN, *Chem. Rev.*, 63 (1963) 325.
12 P. E. BELLIVEAU, V. MALLET AND R. W. FREI, *J. Chromatogr.*, 48 (1970) 478.

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SYNTHESIS, ION-EXCHANGE BEHAVIOUR AND COMPOSITION OF TIN(IV) HEXACYANOFERRATE(II)*

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SUMMARY

Tin(IV) hexacyanoferrate(II) as an inorganic ion exchanger has been prepared in thirteen different ways by varying the pH, the mole ratios mixed and the concentration. Exhaustive analytical studies of a sample having a Sn/Fe mole ratio of 3:1 and an ion-exchange capacity of 2.02 mequiv./g dry weight have been made. These include investigation of its stability in different solvents and its composition, pH-titrations, ion distribution studies, thermogravimetry, and X-ray diffraction measurements. On the basis of all these experimental data a tentative approach to the formula of the compound was made. The potentiality of its use as an ion exchanger has been demonstrated by the achievement of some important and difficult separations.

INTRODUCTION

Metal ion complexes formed with ferrocyanides have recently shown very interesting properties as synthetic inorganic ion exchangers[1]. They are easily prepared and are comparatively less prone to the adverse effects of acids[2] and heat than organic ion exchangers. A high exchange capacity and the fact that they can be used for the separation of radioactive waste and fissionable materials[3] with less damage by radiation than their organic counterparts is indicative of their usefulness. The work on these ion exchangers reported so far mostly concerns their preparation, radiochemical properties, crystallography[4] and the adsorption behaviour of alkali metal ions or alkaline earths[5,6]. The ion-exchange adsorption of other ions has received less attention.

Tin(IV) ion exchangers prepared in these laboratories[7–11] have been found to show very good ion-exchange properties and a few very important and difficult separations have been achieved on papers impregnated with these materials[12,13]. So far, no systematic work on tin(IV) hexacyanoferrate(II) seems to have been reported in the literature. The formation of a yellow precipitate on the addition of

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potassium ferrocyanide solution to stannic chloride solution has been mentioned (see MELLOR[14]) but a detailed study of its use as an ion exchanger is lacking. The preparation and study of the properties of this material were undertaken and the results of such a study are summarized here.

EXPERIMENTAL

Reagents

Stannic chloride pentahydrate (Poland) and potassium ferrocyanide (B.D.H. Analar) were used. All other chemicals were of reagent grade.

Apparatus

Spectrophotometric studies were performed on a Bausch and Lomb spectronic-20 colorimeter. pH-measurements were made on Beckman model-G and Elico Model LI-10 pH-meters. A Philips Camera with an X-ray unit was used for the X-ray studies while thermogravimetry was performed with Stanton thermobalance type H-4.

Synthesis

Thirteen samples were prepared by mixing stannic chloride with potassium ferrocyanide solution under different conditions. The gels formed were digested at room temperature for 24 h. The supernatant liquid was then decanted and the gels were washed several times with distilled water to remove the unreacted reagents. They were filtered and dried at 40–45°. The dry gels were immersed in cold water. They broke down to small particles with cracking and slight evolution of heat. The exchanger was washed with hot distilled water at pH 6–7 to get rid of occluded tin ions or ferrocyanide. It was, then, immersed in 1–2 *M* HNO_3 or HCl for 24 h to convert it to the H^+ form. The exchanger in the H^+ form was washed with distilled water to remove excess acid. When the wash solution had a pH of 6–7 the samples were dried at 40–45°. The particles of desired size were then obtained by passing the ion-exchange material through appropriate sieves. The methods of preparation and some important properties of the different samples are summarized in Table I.

RESULTS

In order to prepare an ion exchanger having a high ion-exchange capacity and low solubility, all the samples prepared were studied on a comparative basis. Their ion-exchange capacity, solubility in distilled water, and compositions are given in Table II.

As is clear from Table II, the ion exchange capacity increases with an increase of the ferrocyanide content in the product. However, the gel character improves with an increase in the tin content of the compound. S-11 was, therefore, selected for detailed study.

Properties

Tin(IV) hexacyanoferrate(II) (Sample S-11) is in the form of blue black, hard, shining granules, suitable for column operation.

TABLE I

SYNTHESIS OF DIFFERENT SAMPLES UNDER DIFFERENT CONDITIONS

Sample No.	*Method of preparation*	*Remarks*
S-1	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:1), pH 4	Green product
S-2	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (2:1), pH 4	Blue product
S-3	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:2), pH 4	Blue product which dissolved during washing
S-4	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:3), pH 4	Blue product which dissolved during washing
S-5	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:1), pH 1	Blue product
S-6	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:1), pH 6.9	Brown product having shiny particles
S-7	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:1), pH 9.9 (ammonia)	Brown product which dissolved in 1–2 *M* HCl in 24 h
S-8	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (1:1), pH 13 (NaOH)	Very small white precipitate which dissolved during digestion
S-9	0.02 *M* $SnCl_4$ + 0.02 *M* $K_4Fe(CN)_6$, (1:1), pH 1	Blue product
S-10	0.01 *M* $SnCl_4$ + 0.01 *M* $K_4Fe(CN)_6$, (1:1), pH 1	Blue product
S-11	0.1 *M* $SnCl_4$ + 0.1 *M* $K_4Fe(CN)_6$, (3:1), pH 0.8	Blue black, shiny particles
S-12	0.04 *M* $SnCl_4$ + 0.04 *M* $K_4Fe(CN)_6$, (3:2), pH 1.3	Blue black, shiny particles
S-13	0.02 *M* $SnCl_4$ + 0.02 *M* $K_4Fe(CN)_6$, (2:1), pH 1.6	Blue black, shiny particles

Chemical stability

To test the chemical stability visually, 0.2–0.5 g of the exchanger was kept in 25 ml of various solutions at room temperature with the following results:

(a) In 12 h there was no change with sulphuric acid, nitric acid, formic acid and acetic acid of all possible concentrations; 4 *M* hydrochloric acid, 0.02 *M* sodium hydroxide and 10% aqueous oxalic acid solutions.

TABLE II

A COMPARISON OF THE ION-EXCHANGE CAPACITY, SOLUBILITY AND COMPOSITION OF THE DIFFERENT SAMPLES

Sample No.	*Composition (Sn:Fe) (mole ratio)*	*Ion-exchange capacity mequiv./dry g*	*Solubility in water*	
			mg Sn/50 ml	*mg Fe/50 ml*
S-1	9.2:1	0.60	0.112	0.50
S-2	3.2:1	1.30	0.112	0.60
S-5	3:5	2.29	0.084	0.50
S-6	100:1	0.11	0.120	0.00
S-9	4:5	3.40	0.240	0.60
S-10	4:5	2.95	0.056	0.70
S-11	5:2	2.02	0.068	0.50
S-12	7:5	2.5	0.120	0.45
S-13	5:2	1.97	0.068	0.70

(b) In 15 min the exchanger dissolved in concentrated hydrochloric acid and 10% ammonium hydroxide.

A detailed study of the chemical stability of the exchanger in water and acids was made as follows:

It was first washed with hot water to remove any tin ions or ferrocyanide remaining adsorbed on the beads. 500 mg of this washed material were then refluxed with 50 ml of distilled water at different time intervals ranging from 1 to 8 h. On quantitative determination of tin and iron in all the filtrates it was noticed that there was a gradual increase in the amount of the dissolved ion exchanger in all cases and after heating for 8 h the exchanger had dissolved almost completely, giving a turbid blue solution. The solubility was determined in different solvents by taking 500 mg of the exchanger in 50 ml of the solvent, and refluxing it with an air condenser for 1 h in each case. After cooling, the solution was filtered and the tin and iron in the filtrate were determined spectrophotometrically by the procedures given below:

Determination of tin. 25 ml of the filtrate were distilled[15] to separate tin from iron. The distillate was diluted to 100 ml with distilled water in a standard flask. 0.5 ml (2:1) sulfuric acid and 5 ml conc. nitric acid were added to a 25 ml portion of the dilute distillate in a 100 ml beaker which was covered with a watch glass and heated to evaporate the liquid completely to dryness. The residue was then cooled and tin was determined with phenyl fluorone as usual[16] against a blank prepared in an identical manner.

Determination of iron. 10 ml of the filtrate were heated in a beaker with 1 ml of conc. H_2SO_4 and 1 ml of conc. HCl to decompose ferrocyanide and cyanide ions. The volume was reduced to about 1 ml and the solution was finally evaporated to dryness. The residue was taken up in a little distilled water and was transferred to a 10 ml standard flask. Iron was determined with 1,10-phenanthroline against a blank[17].

The results of the solubility determinations in water and acids are given in Table III.

Composition

200 mg of the exchanger were heated with 10 ml of H_2SO_4 and 50 ml of HCl. First the solution was blue (ferrocyanic acid) then it turned yellow. It was cooled and stannous chloride solution (60 g $SnCl_2$ in 600 ml HCl and 400 ml H_2O) was added

TABLE III

SOLUBILITY OF TIN(IV) HEXACYANOFERRATE(II) IN WATER AND ACIDS

Solvent	*Amount of tin dissolved in 50 ml (mg)*	*Amount of iron dissolved in 50 ml (mg)*
Distilled water	0.07	0.50
0.1 *M* HCl	0.42	1.75
0.1 *M* HNO_3	0.11	0.35
0.5 *M* HNO_3	0.19	0.35
1 *M* HNO_3	0.32	0.58
0.1 *M* H_2SO_4	0.38	1.58

TABLE IV

COMPOSITION OF TIN(IV) HEXACYANOFERRATE(II)

Wt. of the exchanger (mg)	*Volume of 0.1 N $K_2Cr_2O_7$ used for iron (ml) (a)*	*Volume of 0.1 N $K_2Cr_2O_7$ used for iron and tin (ml) (b)*	*Volume of 0.1 N $K_2Cr_2O_7$ used for tin only (ml) (b−a)*	*Molar ratio (Sn:Fe)* $\left[\frac{b-a}{2} : a\right]$
200	2.25	15.00	12.75	2.7:1
200	2.30	15.95	13.65	2.9:1
200	2.35	14.35	12.00	2.5:1
200	2.35	15.35	13.50	2.9:1
200	2.35	15.40	13.55	2.9:1

dropwise to the cold solution until the colour of iron was no longer evident. The excess of $SnCl_2$ was destroyed with mercuric chloride (saturated solution) as usual. 15 ml of a phosphoric acid–sulphuric acid mixture (150 ml H_3PO_4 + 150 ml H_2SO_4 diluted to 1000 ml) were then added and the volume was made up to about 200 ml with distilled water. Iron was determined by titration against a $K_2Cr_2O_7$ solution using diphenylamine as indicator[18].

For the simultaneous determination of tin and iron in the exchanger 200 mg were dissolved in the sulphuric acid–hydrochloric acid mixture as above. 10 ml of conc. H_2SO_4, 100 ml of conc. HCl and about 2–3 g of test lead in a 500 ml conical flask were added to this solution. The volume was made up to 300 ml with distilled water and the solution was heated for 1 h in an atmosphere of CO_2. It was then cooled to 10° in an ice bath. Tin and iron in the reduced state were titrated against 0.1 *N* $K_2Cr_2O_7$ using diphenylamine as the indicator[19]. The total quantity of tin and iron in the exchanger was thus determined. The amount of tin was then obtained by subtracting the quantity of iron from the total amount of tin and iron. The results for sample S-11 are summarized in Table IV.

Ion-exchange capacity

The gel was found to have cation-exchange properties. Its ion-exchange capacity was therefore determined by testing different mono- and bivalent metal ions in the usual manner[7,8]. A column was prepared in a glass tube of I.D. 6 mm and the flow rate of the effluent was kept at 10–12 drops (approx. 0.5–0.6 ml) per min. Table V summarizes the results.

TABLE V

ION EXCHANGE CAPACITY OF TIN(IV) HEXACYANOFERRATE(II)

Metal ion	*Capacity (mequiv./g dry exchanger)*
Na(I)	2.02
K(I)	2.08
Ba(II)	2.028
Mg(II)	1.88

Titration curve

Several 250 ml conical flasks, each containing 0.5 g of the exchanger, were taken. 0.1 *M* sodium hydroxide and 0.1 *M* sodium chloride were added in different ratios keeping the final volume at 100 ml in all cases. The flasks were kept for 3 days at 25°±1° with intermittent shaking. After attainment of equilibrium the pH values of all the solutions were measured. The results are shown in Fig. 1.

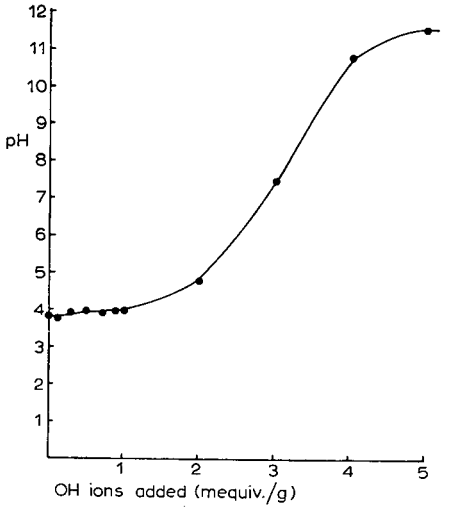

Fig. 1. Titration curve for stannic ferrocyanide.

Distribution coefficients

The usefulness of this exchanger for analytical separations was examined by determining the distribution coefficient with different metal ions in distilled water and 0.1 *M* nitric acid. The following method was used:

2 ml of the metal solution and 98 ml of either water or 0.1 *M* nitric acid were taken in a 250 ml conical flask. The concentration of the solution was adjusted so that the amount of the metal did not exceed 3% of the total capacity of the exchanger[20]. Then 0.5 g of the exchanger in H^+ form was added. The flask was kept at room temperature (25°±1°) for 48 h with occasional shaking. 40 ml of this solution were titrated against 0.002 *M* EDTA solution and K_d values of the metal ion were determined using the formula:

$$K_d = [M_R]/[M_{Sol.}]$$

where $[M_R]$ = amount of the metal ion adsorbed per gram of the exchanger,

$[M_{Sol.}]$ = amount of the metal ion left per unit volume of the solution.

Sodium and potassium were determined in the solution using radioactive tracers. Table VI summarizes all the K_d values.

Thermogravimetry

The results of thermogravimetric analysis of sample S-11 are given in Fig. 2. To check the effect of heat on the colour of the exchanger and on its ion-exchange

TABLE VI

DISTRIBUTION COEFFICIENTS OF SOME METAL IONS

Metal ion	*Compound used*	K_d *value*	
		In water	*In 0.1 M* HNO_3
As(III)	Arsenite	0.0	0.0
Pb(II)	Nitrate	Total adsorption	1693
Cd(II)	Chloride	Total adsorption	1600
Cu(II)	Sulfate	2520	2520
Al(III)	Chloride	Total adsorption	236.4
Ga(III)	Chloride	1680	176
Ni(II)	Sulfate	Total adsorption	840
Co(II)	Chloride	Total adsorption	588.5
Mn(II)	Sulfate	Total adsorption	622.2
Zn(II)	Chloride	Total adsorption	1360.0
Ba(II)	Chloride	704.5	19.4
Sr(II)	Nitrate	1465.2	9.5
Ca(II)	Nitrate	484	0.0
Mg(II)	Nitrate	2470	5.3
K(I)	Chloride	52.6	17.4
Na(I)	Chloride	20.6	—
In(III)	Sulfate	21.4	0.0
Y(III)	Nitrate	8800	362.5
Fe(III)	Alum	156.9	10.9
Bi(III)	Nitrate	Total adsorption	120.0
Sm(III)	Chloride	1905.2	66.6
Nd(III)	Chloride	813.3	8.5
Pr(III)	Chloride	486.3	10.9

capacity 1 g portions of S-11 were heated at 100, 200, 300 and 400° for 1 h in each case. Capacities of all the samples after heating were determined in the usual way. The results are shown in Table VII.

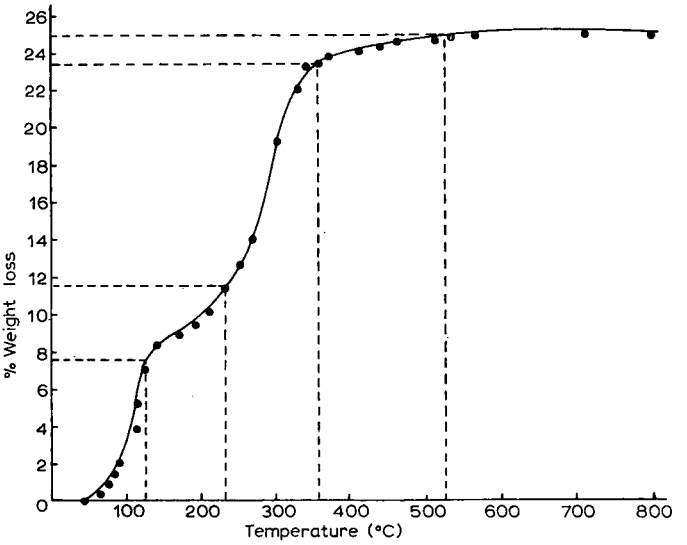

Fig. 2. Thermogram for stannic ferrocyanide.

TABLE VII

EFFECT OF HEAT ON THE EXCHANGER

Temperature (°C)	*Colour of the beads*	*Capacity (mequiv./g)*
Room temp.	Blue black	2.02
100	Blue black	2.02
200	Dirty black	0.60
300	Brownish black	0.02
400	Brown	0.01

X-ray diffraction studies

X-ray diffraction studies showed that tin(IV) hexacyanoferrate(II) is slightly crystalline at room temperature. The crystalline character increases as the temperature is raised, and at 400° a good X-ray pattern is observed.

DISCUSSION

From Tables I and II it is clear that in highly acidic solutions (pH 1) the molar ratio of tin and iron in the gel depends upon the volume ratio of mixing. At higher pH values (pH 4–7) the amount of tin present is always greater than the iron content of the sample. It is probably due to the formation of some tin hydroxide at the higher pH values. This inference is supported by the fact that the precipitates formed at pH 6.9 show Sn:Fe ratio as 100:1. Moreover the samples prepared at pH 10 and at pH 13 dissolve easily in acid solutions. On drying, all the gels turn blue black or prussian blue. This is perhaps due to a redox phenomenon taking place in the system which is indicated by the positive tests obtained for ferro- and ferricyanides in an alkaline solution of the exchanger. When the exchanger is, however, dissolved in a sulphuric acid–hydrochloric acid mixture the solution does not give a positive test for these radicals. This may be due to the decomposition of ferrocyanide and the subsequent oxidation of Fe(II) to Fe(III) by oxygen dissolved in the solution. Such a solution, therefore, gives the test for iron in the trivalent state only. Furthermore, in acidic solutions potassium ferrocyanide forms ferrocyanic acid $[H_4Fe(CN)_6]$. Some of the iron in the bivalent state is oxidized to Fe(III), which with ferrocyanic acid, forms prussian blue $Fe_4[Fe(CN)_6]_3$ in the system, thus resulting in the blue coloured material.

On the basis of chemical and thermogravimetric analysis of the sample and the formula given for titanium(IV) ferrocyanide[21], the following formula for tin(IV) hexacyanoferrate(II) is proposed:

$$[(SnO)_3 \cdot (OH)_3 \cdot HFe(CN)_6 \cdot 3H_2O]_n$$

As shown by the above formula there is only one replaceable hydrogen atom per molecule which is attached to the $Fe(CN)_6^{4-}$ group. This is suggested by one inflection point observed in the titration curve of the exchanger (Fig. 1). The pyrolysis curve for stannic ferrocyanide (Fig. 2) confirms other aspects of the proposed formula. Thus, if this formula is considered to be correct and the percentage weight losses

which are indicated at each break of the curve, are correlated with certain chemical transformations taking place on heating, the following inferences are possible:

(a) Three water molecules per mole of the exchanger are present as water of crystallization. They are lost on heating it up to 125° giving a weight loss of 7.6% (theoretical weight loss of 7.5% due to the removal of 3 H_2O).

(b) Above 125° condensation of the molecule takes place which is indicated by a further loss of 4% in weight of the exchanger at 230°. This loss corresponds to two water molecules which are removed from the structure (theoretical total weight loss of 12.4% at 230° due to the loss of 5 H_2O).

(c) Cyanogen begins to be evolved[22] at 237°, the steeper portion between 230° and 355° therefore corresponds to the decomposition of ferrocyanide and the loss of cyanide as cyanogen. At 355° the total weight loss is 23.4% which closely resembles the theoretical weight loss (23.3%) if three cyanide radicals are assumed to be lost at this temperature.

The portion between 355° and 525° corresponds to one cyanide radical only. It seems therefore that there are only four cyanide radicals available in one molecule of stannic ferrocyanide. Two cyanide radicals must therefore be lost at lower temperatures. This is supported by the fact that a smell of HCN is observed on drying the product.

The titration curve (Fig. 1) shows that when only NaCl is used it releases hydrogen ions and hence a sharp decrease of pH occurs. On the addition of NaOH the pH increases rapidly and above pH 7 the exchanger begins to dissolve. On gradually increasing the volume of NaOH the rate of dissolution and hydrolysis of the exchanger increases and there is a corresponding increase in the pH of the solution. This is perhaps due to the fact that the partial exchange of H^+ with Na^+ slows down the rate of exchange and the release of H^+ ions. No appreciable change in pH is recorded above pH 12.1.

The observed K_d values of metal ions (Table VI) show that this exchanger has a great affinity for Pb, Cd, Cu, Al, Ga, Ni, Co, Mn, Zn, Ba, Sr, Ca, Mg, Y, Bi, Sm, Nd, and Pr in aqueous solutions. Sodium, potassium and indium have low K_d values, and therefore it is possible to separate them from other metal ions. The adsorption of metal ions was also studied in 0.1 M HNO_3 which showed that the K_d value decreased as the hydrogen ion concentration increased. This fact can be predicted if the ion-exchange reaction is defined by the equation:

$$M^{n+}_{aq.} + nHR \rightleftharpoons MR + nH^+$$

and

$$K_d = \frac{[M_R]}{[M^{n+}_{aq.}]}$$

where

$[M^{n+}_{aq.}]$ = concn. of metal ion in the solution;

$[M_R]$ = concn. of metal ion in the exchanger.

If the hydrogen ion concentration is increased the reaction will tend to proceed in the reverse direction and therefore the adsorption of the metal ion on the exchanger

is decreased. Thus rare earths (Sm, Nd, Pr) which show high adsorption in aqueous medium, have very low K_d values in 0.1 M HNO_3. Similarly, Ba, Sr, Ca, and Mg lose their affinity for the exchanger in acid solutions. On the basis of the distribution studies the following separations are possible:

Alkali metals and alkaline earths from Pb(II), Cd(II), Cu(II), Al(III), Ga(III), Ni(II), Co(II), Mn(II), Zn(II).

Arsenic(III), which is taken as arsenite, is not adsorbed on the exchanger beads, obviously due to the cation-exchange behaviour of the material, which completely elutes the arsenite ion. This fact has successfully been utilized for the quantitative separation of arsenic from the metal ions which commonly interfere with this ion such as Pb(II), Cd(II), Cu(II), Ni(II), Co(II), Mn(II) and Zn(II).

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REFERENCES

1 S. KAWAMURA, H. KURAKU AND K. KUROTAKI, *Anal. Chim. Acta*, 49 (1970) 317.
2 D. HUYS AND L. H. BAETSLE, *J. Inorg. Nucl. Chem.*, 26 (1964) 1329.
3 L. H. BAETSLE, D. HUYS AND D. VAN DEYCK, *J. Inorg. Nucl. Chem.*, 28 (1966) 2385.
4 L. H. BAETSLE, D. VAN DEYCK AND D. HUYS, *J. Inorg. Nucl. Chem.*, 27 (1965) 683.
5 V. KOURIM, J. RAIS AND J. STEJSKAL, *J. Inorg. Nucl. Chem.*, 26 (1964) 1761.
6 J. KRTIL, *J. Inorg. Nucl. Chem.*, 27 (1965) 233, 1862.
7 M. QURESHI AND J. P. RAWAT, *J. Inorg. Nucl. Chem.*, 30 (1968) 305.
8 M. QURESHI AND K. G. VARSHNEY, *J. Inorg. Nucl. Chem.*, 30 (1968) 3081.
9 M. QURESHI, R. KUMAR AND H. S. RATHORE, *J. Chem. Soc. (A)*, (1970) 272.
10 M. QURESHI, H. S. RATHORE AND R. KUMAR, *J. Chem. Soc. (A)*, (1970) 1986.
11 M. QURESHI, H. S. RATHORE AND R. KUMAR, *J. Chromatogr.*, 54 (1971) 269.
12 M. QURESHI AND S. Z. QURESHI, *J. Chromatogr.*, 22 (1966) 198.
13 M. QURESHI, I. AKHTAR AND K. N. MATHUR, *Anal. Chem.*, 39 (1967) 1766.
14 J. W. MELLOR, *A Comprehensive Treatise on Inorganic and Theoretical Chemistry*, Vol. VII, Longman's Green, London, 1931, p. 338.
15 E. B. SANDELL, *Colorimetric Determination of Traces of Metals*, III revised ed., Interscience, New York, 1959, p. 856.
16 E. B. SANDELL, *Colorimetric Determination of Traces of Metals*, III revised ed., Interscience, New York, 1959, p. 862.
17 E. B. SANDELL, *Colorimetric Determination of Traces of Metals*, III revised ed., Interscience, New York, 1959, p. 541.
18 W. W. SCOTT, *Standard Methods of Chemical Analysis*, Vol. I, 5th ed., Van Nostrand, New York, 1939, p. 473.
19 I. M. KOLTHOFF AND R. BELCHER, *Volumetric Analysis*, Vol. III, Interscience, New York, 1957, pp. 321–322.
20 H. HAMAGUCHI, R. KURODA, K. AOKI, R. SUGISITA AND N. ONUMA, *Talanta*, 10 (1963) 151.
21 K. H. LEISER, J. BASTIAN, A. B. H. HECKER AND W. HILD, *J. Inorg. Nucl. Chem.*, 29 (1967) 815.
22 C. DUVAL, *Inorganic Thermogravimetric Analysis*, Elsevier, Amsterdam, London, New York, 1953, p. 73.

Notes

CHROM. 5375

A gas chromatographic study of a ternary liquid system

Among the numerous applications of GC, the vast majority are of the analytical type. However, the capabilities of the technique are such that widely different types of physico-chemical studies can also be carried out[1,2]. Among such numerous applications mention may be made of the determination of the activity coefficients of a large number of solutes at infinite dilution in several high-boiling liquids used as stationary phases in GLC columns from measurements of specific retention volumes[3–7]. Although by this method the activity coefficients of hundreds of liquids have been determined, it suffers from the drawback that activity coefficients of solutes can be determined only at infinite dilution and only with the stationary phase in the column as the solvent.

The present study is also concerned with the determination of activity coefficients in liquid systems but by an entirely new technique developed by ARNIKAR *et al.*[8]. They determined the activity coefficients of the components of several binary liquid systems and the results are in good agreement with those obtained by classical methods. The principle is to saturate a quantity of the carrier gas with the vapour over the binary system of known composition and inject a definite volume of it into the column and record the signals for both the components. Similarly, signals are also recorded for the same volume of carrier gas saturated individually with the vapours of the pure liquid at the same temperature. If h is the signal for a component in a solution in which its mole fraction is x and if h_0 is the signal for the vapour of the pure liquid, it has been shown that

$$h/h_0 = \gamma x \qquad (1)$$

where γ is its activity coefficient. This method does not have the limitations of the method mentioned earlier; it can be applied to the determination of activity coefficients of the components of any binary system of volatile liquids of any chosen composition. A further advantage of this method is that it can easily be extended to ternary systems. The present work has been undertaken specifically to emphasize this point, since the activity coefficient data for such systems are scarce. Data have been presented for the activity coefficients of the components in the carbon disulphide, *n*-pentane and diethyl ether system over a wide range of compositions.

Experimental

Analytical grade carbon disulphide, *n*-pentane and diethyl ether were used in the present study and several solutions were prepared in which the mole fraction of carbon disulphide was varied between 0.2 and 0.8 and the rest of the composition was made up of various proportions of *n*-pentane and diethyl ether.

The vapour over each solution was analysed by a Beckman gas chromatograph GC-2. The carrier gas was nitrogen at a flow rate of 40 ml/min. The column was a 1.5-m long, 6-mm I.D. tube packed with Celite (40–60 mesh) loaded for 20% with

polyethylene glycol (PEG-4000) and maintained at 70°. The detector was a katharometer operating at a filament current of 150 mA. The signals were recorded on a Bristol Dynamaster recorder with a sensitivity of 1 mV for full-scale deflection.

The solution was filled into two glass spirals connected in series, each 150 cm long and 7 mm wide and kept in a thermostat at 24.0°. Nitrogen was bubbled through the solution at a rate of 10 ml/min through fine orifices at the bottoms of the spirals. The gas thus saturated with the vapours of the solution was led through the gas sampling valve of the gas chromatograph. This valve had a matched pair of loops (each of 5 ml volume) and at any time one of the loops was in series with the column while the other was open to the atmosphere. The nitrogen saturated with the vapours of the solution was led into the latter loop. On turning the valve handle through a right angle, the two loops exchanged positions and the vapours enclosed in the loop were led by the carrier gas into the column. The signals for the various components were then recorded. The signals for the saturated vapours of the pure liquids were similarly recorded, by taking them individually in the spirals. The column packing was so chosen that the peaks for the various components were sharp, so that the

TABLE I

ACTIVITY COEFFICIENTS IN TERNARY LIQUID SYSTEM OF CARBON DISULPHIDE, *n*-PENTANE AND DIETHYL ETHER

Temperature: 24°. Peak height in number of divisions of chart paper.

Carbon disulphide			*n-Pentane*			*Diethyl ether*		
Mole fraction	*Peak height*	*Activity coefficient*	*Mole fraction*	*Peak height*	*Activity coefficient*	*Mole fraction*	*Peak height*	*Activity coefficient*
1.00	101	1.00	1.00	91	1.00	1.00	72	1.00
0.80	93	1.15	0.20	39	2.14	0.00	0	—
0.80	92	1.14	0.00	0	—	0.20	30	2.08
0.60	80	1.32	0.40	52	1.43	0.00	0	—
0.60	79	1.30	0.20	31	1.70	0.20	28	1.95
0.60	78	1.29	0.00	0	—	0.40	44	1.53
0.40	64	1.58	0.60	69	1.26	0.00	0	—
0.40	62	1.54	0.40	52	1.43	0.20	27	1.88
0.40	62	1.54	0.20	32	1.76	0.40	45	1.56
0.40	61	1.51	0.00	0	—	0.60	55	1.27
0.20	39	1.93	0.80	79	1.09	0.00	0	—
0.20	38	1.88	0.60	66	1.21	0.20	26	1.81
0.20	37	1.83	0.40	52	1.43	0.40	45	1.56
0.20	36	1.78	0.20	30	1.65	0.60	55	1.27
0.20	36	1.78	0.00	0	—	0.80	65	1.13

peak height was proportional to the signal without any appreciable error. The activity coefficient was then calculated in each case by eqn. 1.

Table I presents data for the activity coefficient of all the three components at various compositions of the solution.

Results and discussion

The data in Table I show that the activity coefficients of the various compo-

nents in the ternary system can be determined over wide ranges of concentrations of each component by this new GC technique. The method is simple and can easily be adopted for the study of any multicomponent system of volatile liquids.

For obtaining accurate results, it is necessary to evaluate the signals precisely. This can readily be done by measuring the peak heights when the peaks are narrow and sharp as in the present case. In other cases, one has to resort to more accurate methods for correlating the peaks with the signal strength such as by using disc integrators for measuring peak areas. By such means the utility of the method under present discussion can be improved.

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1 J. C. GIDDINGS AND K. L. MALLIK, *Ind. Eng. Chem.*, 59 (4) (1967) 18.
2 A. V. KISELEV AND Y. I. YASHIN, *Gas-Adsorption Chromatography*, (Translated from Russian by J. E. S. BRADLEY), Plenum Press, New York, London, 1969, p. 104.
3 A. I. M. KEULMANS, *Gas Chromatography* 1st ed., Reinhold, New York, 1957, p. 171.
4 S. KENWORTHY, J. MILLER AND D. E. MARTIRE, *J. Chem. Educ.*, 40 (10) (1963) 541.
5 A. J. B. CRUICKSHANK, B. W. GAINEY AND C. L. YOUNG, *Trans. Faraday. Soc.*, 64 (2) (1968) 337.
6 A. KWANTES AND G. W. A. RIJNDERS in D. H. DESTY (Editor), *Gas Chromatography*, Butterworth, London, 1958, p. 125.
7 C. J. HARDY, *J. Chromatogr.*, 2 (1959) 490.
8 H. J. ARNIKAR, T. S. RAO AND A. A. BODHE, *J. Chem. Educ.*, 47 (1970) 826.

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Die gaschromatographische Trennung von 2-Methylbutanol-1 und 3-Methylbutanol-1 beim im Gerbertest verwendeten Amylalkohol

Zur Bestimmung des Fettgehaltes der Milch wird in der Analytik der Milchwirtschaft seit etwa achtzig Jahren laufend die Gerbermethode angewendet. Die Eigenschaften des bei dieser Methode verwendeten Amylalkohols wirken sich auf deren Genauigkeit aus. Unter anderem spielt hier auch die Struktur des Amylalkohols eine nicht unwesentliche Rolle. Das Gemisch 2-Methylbutanol-1 und 3-Methylbutanol-1 bildet die Grundkomponente des für die Gerbermethode geeigneten Amylalkohols. Die Anwesenheit sekundärer, tertiärer, eventuell auch anderer Alkohole und ihr Verhältnis zum 2-Methylbutanol-1 und 3-Methylbutanol-1 beeinflussen die Genauigkeit der Methode[1]. Im Hinblick darauf ist es vorteilhaft die Struktur des Amylalkohols mittels der Gaschromatographie zu bestimmen.

Bei bisherigen Untersuchungen über den Einfluss der Amylalkohol-Qualität auf die Genauigkeit der Gerbermethode mittels Gaschromatographie wurden die obenerwähnten zwei wichtigsten Alkohole (2-Methylbutanol-1 und 3-Methylbutanol-1) gewöhnlich gemeinsam bestimmt, da die Bedingungen der chromatographischen Analyse, insbesondere die Anwendung stationärer Phasen wie Carbowax 1500, Carbowax 400, Silicon E 301 usw. ihre Trennung nicht ermöglichten[2–8].

COPIUS PEEREBOOM[9] erwähnt die Möglichkeit der Trennung der genannten Alkoholzweiergruppe mittels der Glyzerylmonostearatsäule, die auch EISSES[10] bei seiner Untersuchung auf Reinheit des Amylalkohols anwendet. Beide berufen sich dabei auf die ursprüngliche Methode von ROOS UND VERSNEL[11]. Jedoch auch in den erwähnten Fällen treten im Hinblick auf die Konzentrationsunterschiede im Gemisch der im Amylalkohol vorkommenden Alkohole, Schwierigkeiten bei der vollkommenen Trennung der erwähnten Alkoholzweiergruppe auf und das, trotz Verwendung einer 5 m langen Säule.

Die vorliegende Arbeit beschäftigt sich mit dem Studium der Möglichkeiten der gaschromatographischen Trennung des 2-Methylbutanol-1 und des 3-Methylbutanol-1 im Hinblick auf ihre Anwesenheit im Gemisch mit den übrigen Alkoholen des beim Gerbertest verwendeten Amylalkohols.

Experimenteller Teil

Das analysierte Material. Der Test wurde mit handelsüblichem Amylalkohol tschechoslowakischer Herkunft, der zur Bestimmung des Fettgehaltes der Milch und Milchprodukte mittels der Gerbermethode in den Molkereibetrieben verwendet wurde, durchgeführt.

Analytische Methoden. Amylalkohol wurde mittels Gaschromatographie in gefüllten sowie Kapillarsäulen unter Verwendung verschiedenpolarer stationärer für die Trennung von Alkoholen empfohlener Phasen analysiert. Für diesen Zweck verwendete man Carbowax 1500, Hallcomid, 1,2,3-Tri-(2-cyanoäthoxy)-propan, und Armeen SD. Die Bedingungen der Analyse sind im Text zu den einzelnen Figuren enthalten.

Die Identifizierung der einzelnen Chromatogrammpeaks wurde gemäss des Standards und auf Grund von Literaturangaben durchgeführt[3,12].

Resultate und Diskussion

Von den getesteten Füllsäulen zeigte sich mit Ausnahme der mit Armeen SD gefüllten Säule keine einzige auch nur für eine beiläufige Trennung des 2-Methylbutanol-1 und 3-Methylbutanol-1 geeignet. Aus diesem Grunde erwähnen wir ihre Resultate nicht.

Auch die Verwendung der Säule mit Armeen SD ergab keine zufriedenstellenden Ergebnisse (Fig. 1). Bei der Applikation von Armeen SD auf der Kapillarsäule zeigte sich eine wesentliche Besserung der Trennung beider Alkohole (Fig. 2) im Vergleich zur Füllsäule.

Beim Vergleich mit der Trennung der erwähnten Alkohole auf der Kapillar-

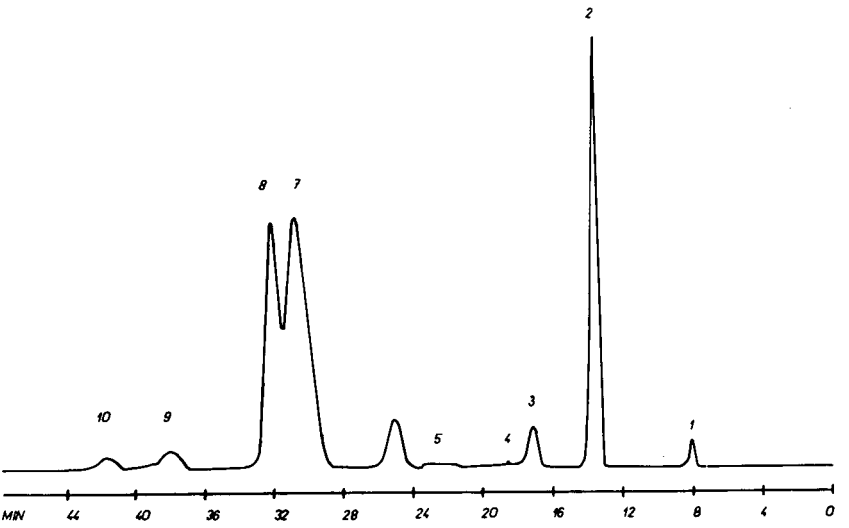

Fig. 1. Chromatogramm des Amylalkohols. Apparat: Fractovap Mod. GB (Carlo Erba, Milan) mit FID. Säule: Glas 263 × 0.3 cm, gefüllt mit 8% Armeen SD auf Chromosorb W, 80–100 mesh. Temperatur, 90°; Trägergas, Stickstoff 1.25 kp/cm²; Dosierung, 0.25 µl. (1))Propanol-1 und Äthanol; (2) 2-Methylpropanol-1; (3) Butanol-1; (6) 2,2-Dimethylpropanol-1; (7) 2-Methylbutanol-1; (8) 3-Methylbutanol-1; (9) Pentanol-1; (4, 5, 10) Nicht identifiziert. Empfindlichkeit: $10^2/4$, Peak 2; $10^2/8$, Peaks 1, 3, 4, 5, 6, 9, 10; $10^2/256$, Peaks 7, 8.

säule mit Igepal CO 210, Igepal CO 530 oder ihrer Gemische[13], Armeen SD (Lit. 12) und der von uns angewandten Methode bei Benützung von Armeen SD ergab sich, dass die Trennung der beiden Alkohole mit unserer Methode günstiger war und dass, sowohl was die Zeit, als auch was die Zusammensetzung und die verschiedenartigen Konzentrationen dieser sich im für den Gerbertest bestimmten Amylalkoholgemisch vorkommenden Alkohole betrifft. Eine weitere Verbesserung bedeutet auch die Symmetrie der Elutionspeaks des Chromatogramms. Das gleiche gilt auch hinsichtlich der beschriebenen Trennung der Alkoholzweiergruppe auf der Füllsäule mit "Tide" (Lit. 14).

Die Symmetrie der chromatographischen Peaks bei Anwendung der Kapillarsäule mit Armeen SD wird auf die Zugabe von Tetrahydroxyäthylendiamin zurückgeführt (Fig. 2).

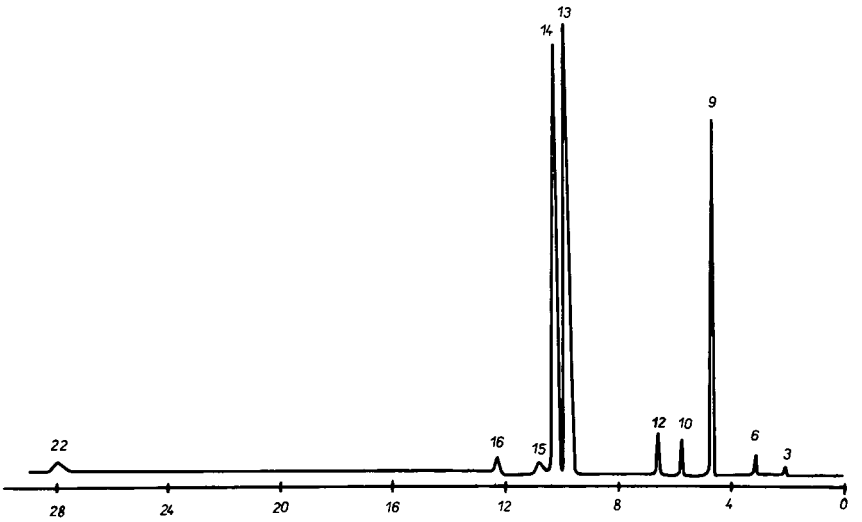

Fig. 2. Chromatogramm des Amylalkohols. Apparat: Perkin Elmer Mod. F-11 mit FID. Kolonne: Kapillarkolonne 20 m × 0.25 mm, imprägniert mit 95 mg Armeen SD + 5 mg Tetrahydroxyäthylendiamin, Lösungsmittel CH_2Cl_2 + $CHCl_3$ (2:1). Temperatur, 60°; Trägergas, Stickstoff 0.4 kp/cm²; Dosierung, 0.2 µl (Teiler 1:50). (3) Äthanol; (6) Propanol-1; (9) 2-Methylpropanol-1; (10) Butanol-1; (12) 2,2-Dimethylpropanol-1; (13) 2-Methylbutanol-1; (14) 3-Methylbutanol-1; (15) Nicht identifiziert; (16) Pentanol-1; (22) Hexanol-1. Empfindlichkeit: 5 für Peaks 3, 6, 9, 10, 12, 16, 22; 50 für Peaks 13, 14.

Versuche mit Applikation von Carbowax 200 in der Füllsäule, die mittels der beiden hier behandelten Alkohole in Whisky, Branntweinen oder Banan festgestellt wurden[15,16], konnten in unserem Fall kein Resultat liefern.

Aus den vorliegenden Resultaten folgt, dass sich bei der Trennung von 2-Methylbutanol-1 und 3-Methylbutanol-1 die Verwendung der Kapillarsäule mit Armeen SD und Tetrahydroxyäthylendiamin als vorteilhaft erwiesen hat. Diese Säule kann bei der komplexen chromatographischen Untersuchung der Struktur des Amylalkohols, der zur Bestimmung des Fettgehaltes der Milch mittels der Gerbermethode benützt wird, verwendet werden. Die Applikation der hier beschriebenen Methode beim Testen der Eignung in der Tschechoslowakei bei der Gerbermethode verwendeter Amylalkohole wird an anderer Stelle beschrieben werden[17].

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1 R. J. VALIANT UND B. L. HERRINGTON, *Milchwiss.*, 14 (1959) 275.
2 H. HÄNNI, *Mitt. Lebensm. Hyg.*, 51 (1960) 515.
3 H. HÄNNI UND W. RITTER, *Milchwiss.*, 16 (1961) 24.
4 P. JAMOTTE, *Lait*, 46 (1966) 255.
5 H. L. J. C. HENDRICK, A. D. C. HUYGHEBAERT UND R. L. DEMETER, *17th Int. Dairy Congr.*, B (1966) 133.
6 K. P. ANDERSEN UND K. J. JØRGENSEN, *Beretn. Statens Forsøgsmejeri*, 132 (1961) 140.

7 A. SVENSEN UND K. STEINSHOLT, *Milchwiss.*, 18 (1963) 530.
8 A. FRICKER UND F. WOERNER, *Kieler Milchwiss. Forschungsber.*, 13 (1961) 373.
9 J. W. COPIUS PEEREBOOM, *Neth. Milk Dairy J.*, 17 (1963) 81.
10 J. EISSES, *Neth. Milk Dairy J.*, 19 (1965) 165.
11 J. B. ROOS UND A. VERSNEL, Rijkszuivelstation, nicht publiziert (von Lit. 9).
12 *Barber-Colman Co., Bull.* F-9791 (AE 6007) (1963).
13 R. D. SCHWARTZ UND R. G. MATHEWS, *J. Chromatogr. Sci.*, 7 (1969) 593.
14 P. PORCARO UND V. D. JOHNSTON, *Anal. Chem.*, 33 (1961) 361.
15 D. D. SINGER, *Analyst*, 91 (1966) 790.
16 K. E. MURRAY, J. K. PALMER, F. B. WHITFIELD, B. H. KENNETT UND G. STANLEY, *J. Food Sci.*, 33 (1968) 632.
17 V. PALO, J. VALČÍKOVÁ UND J. HRIVŇÁK, *Prům. Potravin.*, 8 (1971) im Druck.

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Parent hydrocarbon determination by means of gas chromatography

During recent years gas chromatography (GC) has even been favoured as a method for solving problems concerning the constitution of organic compounds. Particularly, its use as a fingerprint determination of a compound enabling one to gain very rapid and reliable information with respect to the character of a given sample is possible.

Among the many methods used for determining the carbon structure in classical organic analysis, there are two methods which especially are suitable for adaptation in the form of reaction GC:

(1) the mild, but not in the least universal, method of catalytic hydrogenation;

(2) a more universal but not fully unambiguous (with respect to reaction products) reduction method by means of zinc dust.

The first method was used by BEROZA[1] who hydrogenated the substance on Pt-group metals, reduced on some suitable support, in an electrically heated aluminium tube.

We have used the second method based on a method of VON BAEYER[2] which is more than a hundred years old and which has also been modified for the submicro scale[3]. GASPARIČ[5] has used CLAR's low-temperature modification[4] of this method in connection with paper chromatography for the study of the constitution.

In this study a TPGC apparatus, manufactured by Development Laboratories and Workshops in Olomouc, and equipped with a TC detector, was used. The dual coiled column system was filled with 20% Apiezon L–Chezasorb. In front of the packed column was inserted a reactor[8] made of Supremax glass filled with a mixture of distilled zinc dust (min. 99.9%) and diatomaceous support; this was heated with a resistance oven forming the outer shell of the reactor tube. The sample was introduced into the reactor by means of a microsyringe. Hydrogen was used as carrier gas. The substances leaving the column were identified on the basis of their retention times as well as by chemical reactions[6].

It was found that under similar conditions, aliphatic as well as aromatic halogen derivatives, and alcohols, aldehydes and ketones of all classes are quantitatively converted into their corresponding parent hydrocarbons. With substances containing other functional groups, no uniform conversion takes place under the conditions mentioned below and therefore these groups are the object of a further study.

In order to obtain a quantitative conversion the following parameters have proved to be critical.

The reactor temperature

The optimum working temperature is near the melting point of zinc (from 410 to 420°). In the case where the zinc is deposited in greater amounts on the diatomaceous support one can work with even higher temperatures. It is sometimes very convenient to use higher temperature because with such temperatures melting takes place and results in the renovation of the zinc surface. It has already been proved

that the conversion in the exhausted reactor can be increased by extending the time interval between individual doses.

Flow rate of the carrier gas

The conversion increases with decreasing flow rate (longer retention time of a sample in the reactor, thus greater chance of establishing perfect thermodynamic equilibrium). A flow rate of 20 ml H_2/min was chosen as optimum.

The amount of the sample and the quantity of zinc in the reactor

It was found that the degree of conversion depends on the ratio between the amount of sample and the amount of zinc in the reactor (and thus the dimensions of the reactor). In this case for the conversion with the groups of substances already mentioned to be quantitative, the other given optimum conditions must be fulfilled and it is necessary that the reactor contains a 7×10^4 greater amount of zinc than would be necessary as a minimum to induce quantitative reduction. With such an increase in the size of the reactor the gas hold space also increases which results in an extension of the peak width. For 5-μl doses, which were chosen with respect to their reproducibility and the possibility of quantitative evaluation of the degree of conversion (planimetry of the peak areas), a reactor of the size 20 $\times$ 2.2 cm proved to be best. With this reactor 100% conversion still takes place after the introduction of several tens of 5 μl samples and the introduction of several thousand 0.1–0.2 μl samples (for qualitative studies).

Quality of the zinc support

The choice of the reactor packing is influenced by the high cracking efficiency of some supports[7]. The use of the 40% (w/w) Zn/Chezasorb packing of the reactor at 424° resulted, even with chlorobenzene (from which benzene is developed and characterized by its appreciable stability), in the formation of 3–5% of pyrolysis products in addition to 95–97% of benzene. The pyrolysis products only disappear from the chromatogram when the chlorobenzene conversion ceases to be quantitative. With the use of Chromaton N (a product of Lachema) the formation of low-molecular cracking fragments was depressed. Even the glass walls of the reactor show some slight pyrolytic and reaction activity[7,8].

Thus a cylindrical glass reactor (20 $\times$ 2.2 cm) filled with 90% Zn/Chromaton N (w/w) at 420° and with a flow rate of 20 ml/min proved to give the best results.

In addition to the usual advantages of GC the method investigated has the following ones:

(1) The reactor packing is easily prepared and the life-time of the reserve packing is unlimited.

(2) The packing is insensitive to catalyst inhibitors. The zinc need not be activated and any hydrogen which is adsorbed in the packing plays no active role in the reaction.

(3) Low-molecular weight reactive fragments (H_2O, H_2S, HX) are intercepted in the reactor, thus preventing corrosion of the column and the detector. Water is retained in the reactor at temperatures above 350°. In this way a promising method

for the analysis of aqueous solutions of ethereal oils and essences and dilute aqueous solutions in general is suggested.

(4) There is the possibility of using larger samples for subsequent spectroscopic analysis.

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1 M. BEROZA, *Anal. Chem.*, 34 (1962) 1801.
2 A. VON BAEYER, *Ann. Chem.*, 140 (1866) 295.
3 A. TREIBS AND H. STEIMMETZ, *Ann. Chem.*, 506 (1933) 171.
4 E. CLAR, *Chem. Ber.*, 72 (1939) 1645.
5 J. GASPARIČ, *Mikrochim. Acta*, 1-2 (1966) 288.
6 J. T. WALSH AND C. MERRITT, JR., *Anal. Chem.*, 32 (1960) 1378.
7 M. VERZELE, K. CAUWENBERGHE AND J. BOUCHE, *J. Gas Chromatogr.*, 5 (1967) 114.
8 J. GRÚZ, L. ČÁP AND Z. STRÁNSKÝ, *Coll. Czech. Chem. Commun.*, in press.

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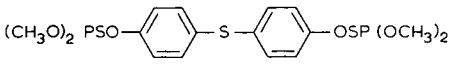
Alkali-flame gas chromatography of Abate*

The insecticide Abate® (O,O,O′,O′-tetramethyl-O,O′-thiodi-*p*-phenylene phosphorothioate) (I) is one of the newer chemicals proven to be effective in the control of mosquito larvae[1,2]. A number of analytical methods for residue determinations of this chemical have already been published, the majority of which use the gas chromatograph. Flame ionization[3], electron capture[4], and flame photometric detectors[4–6] have all been employed in these chromatographic procedures. Because of the limited sensitivity of flame ionization and the loss of specificity of electron capture, the flame photometric detector, operated in either the phosphorus or sulfur mode, appears to be the detector of choice. DALE AND MILES[4] and BOWMAN *et al.*[5] report levels of sensitivity at 2–5 ng when operating in the phosphorus mode and 30–50 ng when using the sulfur mode. However, both indicate the necessity of adapting a water cooling system to the detector to prevent damage while operating at the high temperatures required. A current model of the flame photometric detector is now available which is designed to operate at higher temperatures and does not require a water cooling system.



In addition, BOWMAN *et al.*[5] found it difficult to maintain the conditioned state of the column, necessitating the injection of 50 ng or more of a standard solution alternately with the unknowns being analyzed. However, DALE AND MILES[4], using an XE-60 column of 2-in. length, had no problem in maintaining the conditioned state. SHAFIK[6] developed the technique of hydrolysis of Abate to 4,4′-thiodiphenol and treating this compound with trimethylchlorosilane and hexamethyldisilazane to form a silylated derivative. This technique allows the conditioned state of the column to be easily maintained with operation of the detector at a lower temperature (thus omitting the use of a cooling system) and produces a level of sensitivity of 5–10 ng when operating in the sulfur mode.

Since many laboratories may lack the photometric detector, the following investigation was made to develop a rapid, specific, sensitive method of analysis for Abate using the relatively inexpensive alkali-flame detector.

Materials and methods

A Varian Aerograph, Model 1700, equipped with an alkali-flame detector and an aluminum column, 1/8 in. O.D. × 10 in., packed with 2.5% E-301 + 0.25% EPON 1001 on 80/100 mesh Gas-Chrom W (AW-DMCS)HP was used.

Operating parameters were: injector, column and detector temperature 235°; flow rates, nitrogen 50 ml/min, air 120 ml/min, hydrogen 20 ml/min; electrometer

* Mention of a proprietary product does not constitute endorsement by either the Public Health Service or the Agency for International Development.

attenuation, $2 \cdot 10^{-10}$ A/mV. Air and nitrogen were supplied by pressure-regulated cylinders, and hydrogen was produced from an Elhygen Hydrogen Generator. All Abate standards were prepared in diethyl ether.

The column was preconditioned for 24 h, after which five injections of 1 $\mu g/\mu l$ were made at intervals of 10 min each. The column was considered ready for use when injections of 20 ng every 10 min produced identical responses of 10% scale. Retention time for the chemical was 3 min.

Results

We encountered the same problem of column conditioning as previously reported[5] and, in the manner suggested by these authors, alternating injections of the unknowns with a 20 ng standard eliminated the problem. The response of the detector was linear to 200 ng. Water samples, fortified with Abate to produce concentrations of 1.0, 0.1 and 0.01 p.p.m., were extracted by the method of DALE AND MILES[4] and were brought to a final volume of 1 ml in diethyl ether. Recoveries were 97% as determined by the above chromatographic procedure.

The increased sensitivity and specificity of the alkali-flame detector for phosphorus makes it more acceptable for analysis of such chemical containing compounds than either the flame ionization or electron capture detectors. The results of this investigation provide the analyst with a method which is rapid, sensitive, and specific, and closely approaches the results obtainable with the flame photometric detector.

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1 C. S. LOFGREN, J. E. SCANLON AND V. ISRANGURA, *Mosquito News*, 27 (1967) 16.
2 J. B. MOORE AND S. G. BREELAND, *Mosquito News*, 27 (1967) 105.
3 F. C. WRIGHT, B. N. GILBERT AND J. C. RINER, *J. Agr. Food Chem.*, 15 (1967) 1038.
4 W. E. DALE AND J. W. MILES, *J. Agr. Food Chem.*, 17 (1969) 60.
5 M. C. BOWMAN, H. R. FORD, C. S. LOFGREN AND D. E. WIEDHASS, *J. Econ. Entomol.*, 61 (1968) 1586.
6 M. T. SHAFIK, *Bull. Environ. Contam. Toxicol.*, 3 (1968) 309.

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CHROM. 5325

Gas–liquid chromatography and mass spectrometry of various benzodiazepines

Several benzodiazepine derivatives have been shown to have powerful pharmacological and therapeutic effects[1]. Studies concerning the metabolism and tissue distribution of diazepam and three of its known metabolites in correlation with the anticonvulsant effects have been carried out in this laboratory[2–4] and required the availability of specific and precise micromethods for measuring the administered drug and its metabolites.

In previous papers a method for the gas chromatographic (GC) separation and the quantitative analysis of several benzodiazepines, including diazepam and some of its known metabolites, was described[5,6]. It was considered of interest to establish the identity of the GC peaks by using mass spectrometry (MS) coupled with GC. In addition, this report describes an improved GC procedure for benzodiazepine analysis which allows better separation, greater reproducibility and higher sensitivity.

Apparatus

GC analysis was carried out using a Carlo Erba Model GV instrument equipped with a flame ionization detector. The stationary phase used was OV-17, a more polar methyl phenyl silicone derivative which was found to be superior to the OV-1 phase used in the original method[5]. The column was a 2 m column of 3% OV-17 on Gas-Chrom Q (100–120 mesh) contained in glass tubing (2 mm I.D. × 4 mm O.D.). The carrier gas was N_2 at a flow rate of 37 ml/min.

The injection site temperature was 300°, the detector was 270° and the oven was 250°. The LKB Model 9000 instrument, fitted with a 2 m × 2 mm I.D. spiral glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) was employed for combined GC–MS experiments. Helium was used as the carrier gas and all the mass spectra were obtained at 12 eV and 70 eV. Other parameters were: injection site 300°, molecular separators 260°, ion source 290°, accelerating voltage 3 kV, ionizing current 20 μA. Only the 12 eV mass spectra or the 70 eV mass spectra were considered because of their greater simplicity in fragmentation patterns.

Chemicals

Synthesis of 6-chloro-4-phenylquinazoline-2-carboxaldehyde (QCA). 5 g of oxazepam were melted at 200° until steam development ceased (5–10 min). After cooling the compound was filtered and crystallized from benzene. Yellow prisms of QCA showing a m.p. of 177–178° and an 80% yield were obtained. The R_F was 0.42 on TLC using benzene–ethyl acetate (5:2) as development solvent; the I.R. spectrum had a $\nu_{max.}$ at 1728 cm^{-1}; the NMR spectrum showed adsorption at $\delta = 10.3$ (O=C–H). The mass spectrum is represented in Fig. 3.

QCA after crystallization from EtOH gives white crystals of hemiacetal. Both the former and the latter compound are identical to those synthesized by the method of STERNBACH *et al.*[7].

Synthesis of 7-chloro-1-deutero-3-hydro-3-deuteroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (II) (Fig. 1). 0.01 mole of 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-

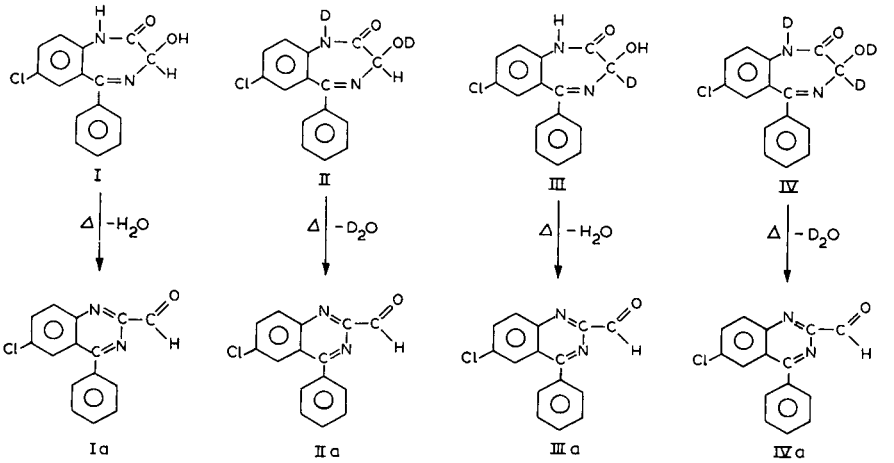


Fig. 1. Structural formulae of the compounds synthesised as described under *Chemicals* in the text.

1,4-benzodiazepin-2-one (I) was dissolved in dry dioxane; an amount of D_2O, slightly exceeding the stoïchiometric value, was added and the solution was shaken for 3–4 h.

The deuterated compound was precipitated by adding D_2O.

Synthesis of 7-chloro-1-hydro-3-deutero-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (III) (Fig. 1). This was prepared according to a slightly modified method from HOFFMAN-LA ROCHE[8].

The mixture of 7-chloro-1-hydro-3,3-dideutero-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 5) having 61% of dideuterated molecules (0.5 mmole), N-chlorosuccinimide (0.5 mmole) and benzoyl peroxide (2.5 mg) was refluxed, with stirring, for 1 h. Solids were removed by filtration and the filtrate was evaporated to a small volume. 0.1 mmole of sodium acetate in 1.9 ml of acetic acid was added and then the solution was heated in a water bath for 2 h; the suspension was evaporated to about half volume, and it was then cooled, and then an equal volume of water was added. The solids were removed by filtration, washed with water and then suspended, under agitation, in a small amount of acetic acid until a crystalline product was obtained.

The acetyl oxazepam obtained was washed with ether; the m.p. was 241–242°. To a suspension of acetyl oxazepam (0.5 mmole) in 2 ml of 95% ethanol, 0.11 ml of 9 *M* NaOH was added at room temperature. At first there was complete solution but this was followed by the formation of a precipitate. The suspension was diluted with 2 ml of water until complete solution and then acidified with acetic acid.

The crystalline oxazepam (III) (Fig. 1) precipitated, and after washing with water it was recrystallized from alcohol; m.p. 203–204°.

Synthesis of 7-chloro-1,3-dideutero-3-deuteroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (IV). This compound was obtained by shaking the compound III with D_2O according to the procedure described above.

Results and discussion

The stationary phase used (OV-17) allows an efficient separation of the benzo-

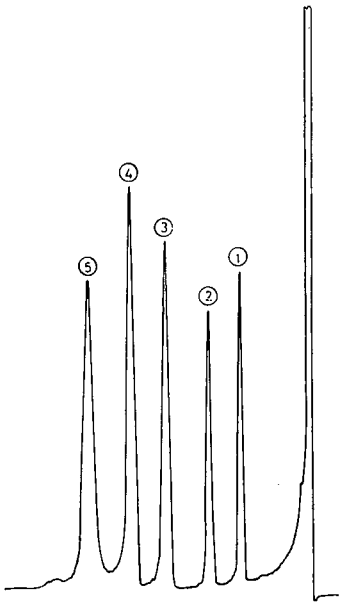


Fig. 2. Gas chromatographic separation of a mixture of five benzodiazepines. 1 = oxazepam; 2 = diazepam; 3 = N-demethyldiazepam; 4 = N-methyloxazepam; 5 = nitrazepam.

diazepines tested as indicated in Fig. 2. The sensitivity as well as the retention time are reported in Table I where a comparison is made between results obtained with OV-1 and OV-17. The identity of the GC peaks was established by MS. The mass spectra obtained after GC separation were analyzed for the following compounds: 7-chloro-1-methyl-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (diazepam); 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (N-demethyldiazepam); 7-chloro-1-methyl-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (N-methyloxazepam); 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (oxazepam); 7-nitro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (nitrazepam). The fragmentation pattern of the examined benzodiazepines and the structural implications of this fragmentation will be published elsewhere. The mass

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIME OF SEVERAL BENZODIAZEPINES USING OV-1 AND OV-17 PHASES

Drug	*Retention time*	
	OV-1	*OV-17*
Oxazepam	3 min (0.25)[a]	2 min 12 sec (0.25)
Diazepam	4 min 10 sec (0.20)	3 min 04 sec (0.20)
N-Demethyldiazepam	4 min 45 sec (0.40)	4 min 40 sec (0.20)
N-Methyloxazepan	6 min 12 sec (1.00)	5 min 44 sec (0.50)
Nitrazepam	8 min 30 sec (0.50)	7 min 12 sec (0.50)

[a] Figures in parentheses = sensitivity in μg (flame ionization detector).

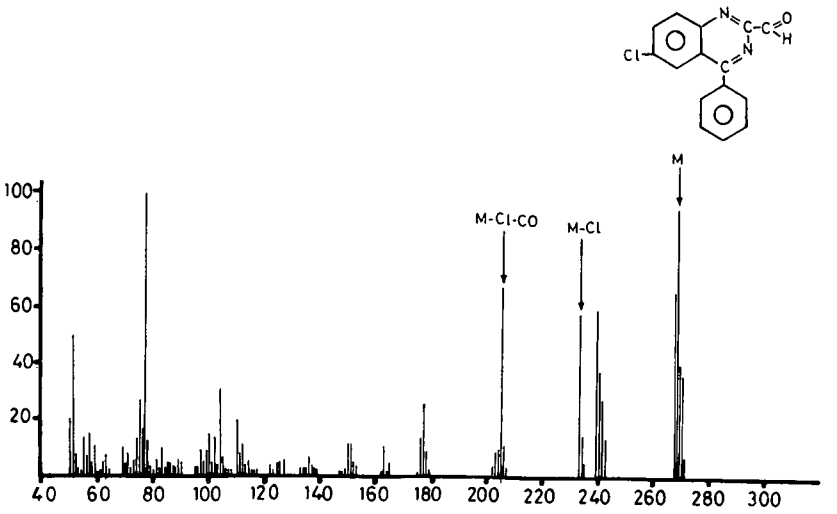


Fig. 3. Mass spectrum of 6-chloro-4-phenyl-2-carboxaldehyde; M = 268, 70 eV.

spectra of the GC peaks show that the structure of the analyzed benzodiazepines was not modified under the experimental conditions used with the exception of oxazepam. The latter compound, as a result of the heating, is rearranged to form 6-chloro-4-phenylquinazoline-2-carboxaldehyde with the loss of an H_2O molecule.

This compound, previously obtained by STERNBACH *et al.*[7] by treating oxazepam with HCl, was obtained in this laboratory as described under *Chemicals* with almost quantitative yields by heating oxazepam at 200°.

Its retention time in GC and its mass spectrum are identical to that obtained with the GC peak of oxazepam. The structure of 6-chloro-4-phenylquinazoline-2-

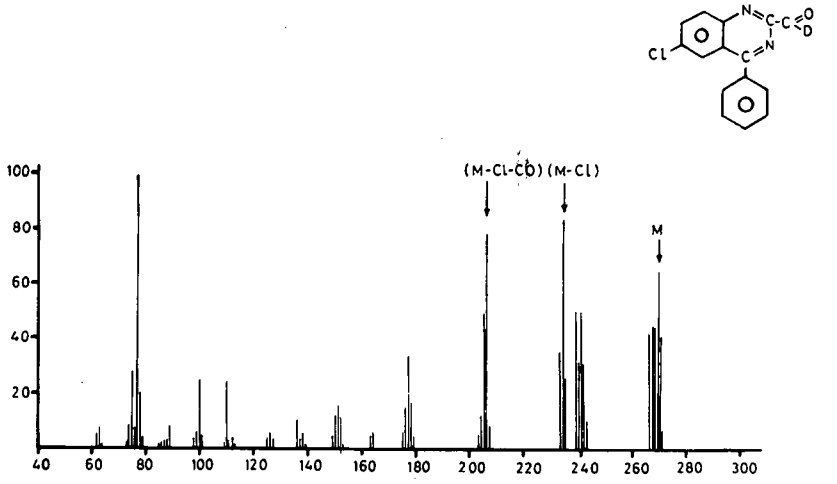


Fig. 4. Mass spectrum of monodeutero-substituted quinazoline carboxaldehyde; M = 269, 70 eV.

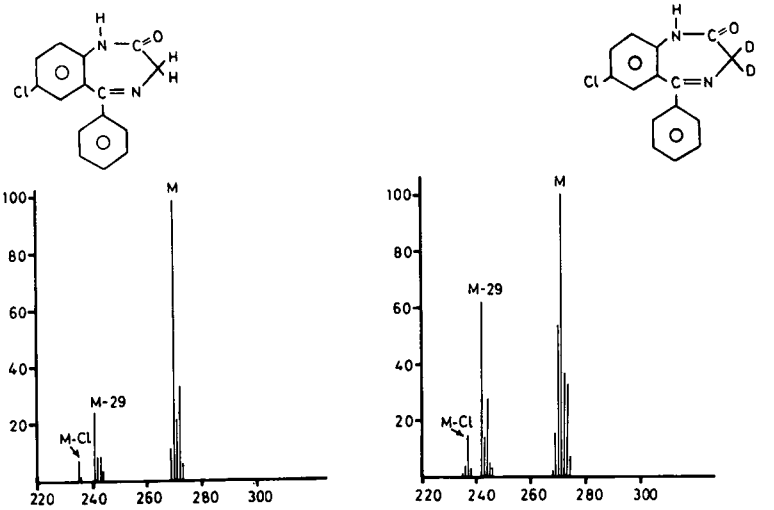


Fig. 5. Mass spectra of N-demethyldiazepam and 3-dideutero-substituted N-demethyldiazepam; M = 270, M = 272, 12 eV.

carboxaldehyde (QCA) has been further confirmed by means of NMR spectroscopy.

In order to investigate the dehydration mechanism of oxazepam under thermolytic conditions, benzodiazepines and deuterium-labelled benzodiazepines were synthesized. The molecules synthesized are shown in Fig. 1. The four compounds I, II, III, IV were transformed into the respective QCAs (Ia, IIa, IIIa, IVa) as described under *Chemicals*, and then analyzed in the mass spectrometer. SCHWARTZ[9] has previously reported the characteristic fragmentations of oxazepam: M, M—H_2O, M—HCO, M—$C_2H_2NO_2$. Some of these, namely M and M—HCO, also occur in the mass spectrum of the QCA derivative. Fig. 3 shows that the loss of HCO is a phenomenon similar to the one observed for oxazepam; the intensity of the molecular peak and the peaks deriving from the loss of chlorine and chlorine plus carbon monoxide are larger than those of the corresponding hydroxylated benzodiazepine (compound I). The QCAs obtained from compounds III and IV had the same mass spectra, whereas the one obtained from oxazepam II exhibited a mass spectrum identical to the unlabelled standard QCA.

TABLE II

DIFFERENCES IN THE FRAGMENTATION PATTERN OF IIa, IIIa, IVa QCAs WITH RESPECT TO THE STANDARD Ia

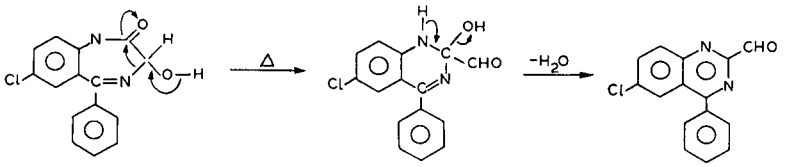
The *m/e* ratios of the compounds IIIa and IVa are shifted by one unit with respect to the standard (Ia) and IIa QCAs.

Formula	*Standard QCA m/e Ia*	*IIa m/e*	*IIIa m/e*	*IVa m/e*
M	268	268	269	269
M — Cl	233	233	234	234
M — Cl — CO	205	205	206	206

Table II reports on several simple fragmentations concerning unlabelled QCA and the aldehydes IIa, IIIa and IVa. The *m/e* ratios of compounds IIIa and IVa are shifted by one unit with respect to the standard and IIa compounds. The amount of deuterium found in the former two compounds (58%) is in agreement with the amount of deuterium (61%) in the starting material 7-chloro-3,3-dideutero-5-phenyl-2H-1,4-benzodiazepin-2-one.

For simplicity, the deuterated entity was calculated by utilizing the peaks corresponding to the molecular ion minus one atom of chlorine (M−Cl).

This finding suggests that one of the hydrogen atoms involved in the dehydration mechanism of the oxazepam molecule is bound to N_1 and the other is part of the hydroxylic group at C_3. As a result of the fact that N_4 is more nucleophilic than N_1 the possible dehydration mechanism may be represented as follows:



The results reported in Table II confirm that the 1-deutero-3-deuteroxy-substituted oxazepam II gives rise to an unlabelled aldehyde IIa.

Therefore, when the 3-deutero- or the 1,3-dideutero-3-deuteroxy-substituted benzodiazepines are used as starting material the same monodeuterated thermolytic transformation products (IIIa, IVa) are obtained.

We wish to thank Dr. T. SALVATORI, SNAM Progetti, San Donato Milanese, for his kind cooperation in running the mass spectra, and Mr. F. MAURI, Ravizza Co., Muggiò, Milan, for the synthesis of 6-chloro-4-phenylquinazoline-2-carboxaldehyde. This work was supported by Contract DHEW PH/NIH/43-67-83.

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1 G. ZBINDEN AND L. O. RANDALL, in S. GARATTINI AND P. A. SHORE (Editors), *Advances in Pharmacology*, Vol. 5, Academic Press, New York, 1967, pp. 213–291.
2 F. MARCUCCI, R. FANELLI, M. FROVA AND P. L. MORSELLI, *Eur. J. Pharmacol.*, 4 (1968) 464
3 F. MARCUCCI, R. FANELLI, E. MUSSINI AND S. GARATTINI, *Eur. J. Pharmacol.*, 9 (1970) 253.
4 F. MARCUCCI, E. MUSSINI, R. FANELLI AND S. GARATTINI, *Biochem. Pharmacol.*, 19 (1970) 1847.
5 F. MARCUCCI, R. FANELLI AND E. MUSSINI, *J. Chromatogr.*, 37 (1968) 318.
6 S. GARATTINI, F. MARCUCCI AND E. MUSSINI, in R. PORTER (Editor) *Gas Chromatography in Biology and Medicine*, Churchill, London, 1969, pp. 161–172.
7 L. H. STERNBACH, E. REEDER, A. STEMPER AND A. J. RACHLIN, *J. Org. Chem.*, 29 (1964) 332.
8 HOFFMANN-LA ROCHE, *South African Pat. No. 667088*, 1967.
9 M. A. SCHWARTZ, F. M. VANE AND E. POSTMA, *J. Med. Chem.*, 11 (1968) 770.

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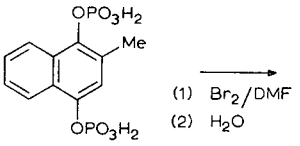
CHROM. 5383

Combined gas–liquid chromatography/mass spectrometry study of the bromine oxidation of Synkavit (2-methylnaphthalene-1,4-diol diphosphate) in ^{18}O-enriched dimethylformamide

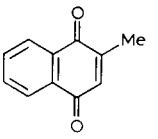
The combination of gas–liquid chromatography (GLC) and mass spectrometry (MS) has proved to be applicable to a wide variety of analytical problems[1–12]. The success of this technique has prompted us to employ it in a re-examination of the bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate (Synkavit, I) in ^{18}O-enriched dimethylformamide as solvent. Both the 2-methyl-1,4-naphthoquinone



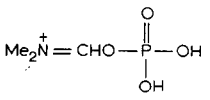
I

(1) Br_2/DMF
(2) H_2O

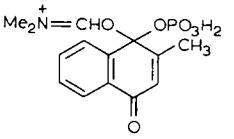
$2H_2PO_4^-$ +



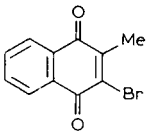
II



III



IV



V

(Menadione, II) and the inorganic phosphate (P_i) products of this reaction were found in the initial study[13] to be isotopically labelled by conversion of each to carbon dioxide and determination of the ^{18}O to ^{16}O ratio by MS. With the combination technique one uses the GLC column to present a few micrograms of sample to the mass spectrometer for determination of the excess ^{18}O content of the molecule itself (molecular ion) or fragment ions thereof. We have recently reported on the determination of the ^{13}C content of amino acids from algae grown on ^{13}C-enriched CO_2 via combined GLC–MS of their TMSi derivatives[11]. It was felt that a comparison of results for the direct (GLC–MS) and the indirect (conversion to CO_2 followed by MS) methods for the determination of ^{18}O content of Synkavit and P_i would be a test of their relative merits and consistency. This was considered particularly worthwhile in view of the conclusions drawn from observing the presence of label in the two products—*i.e.*, that the bromine oxidation proceeded via the intermediates III and

IV to the extent of 26% (P–O bond cleavage) and 11%* (C–O bond cleavage), respectively[13].

Experimental

GLC–MS experiments with 1–2 μg samples were carried out with an LKB Model 9000 instrument. The spiral glass GLC column was 4 ft. × 3 mm I.D.; 3% OV-17 on acid-washed and silanized[4] Gas-Chrom P; 30 ml/min helium carrier gas. Mass spectra were obtained with a 70 eV ionizing potential, a 3.5 kV accelerating potential, a 50 μA filament current, and a 270° ion source temperature.

Phosphoric acid (Merck) and the ^{18}O-enriched acid (obtained from P_i—magnesium phosphate—by ion-exchange) were taken up in bis-trimethylsilyltrifluoroacetamide and allowed to react for 30 min at 45° to form the tri-trimethylsilyl (TMSi) derivative[14]. This compound exhibited a retention time of 6 min (temperature-programmed analysis: initial temperature 80°, 5°/min). MS peak intensity measurements were made on both the molecular ion M (*m/e* 314) and M-15 (*m/e* 299) fragment and for the corresponding isotope signals (*m/e* 316 and 301, respectively). Multiple GLC–MS runs were made and mean values determined. Calculation of the percent excess ^{18}O for the M-15 fragment is given below; the same value was obtained from the molecular ion.

	M-15	
	m/e 299	*m/e 301*
Intensity ^{18}O-enriched $PO(OTMSi)_3$	100	19.0
Intensity reference $PO(OTMSi)_3$	100	13.5
Intensity difference		5.5

$$\frac{5.5}{5.5 + 100} \times 100 = 5.2\% \text{ excess } ^{18}O$$

Intensity measurements and calculations were also made on the M (*m/e* 172) and the M+2 (*m/e* 174) signals of the ^{18}O-enriched 2-methyl-1,4-naphthoquinone and a reference sample. A value of 2.9% excess ^{18}O was obtained.

Results and discussion

GLC of the tri-TMSi phosphate derivative of P_i indicated the sample to be homogeneous, and the compound was identified by its retention behavior and mass spectrum. The value of methods for readily converting inorganic anions to volatile derivatives[14] amenable to GLC and MS is evident. Analysis of the 2-methyl-1,4-naphthoquinone** isolated from the Synkavit oxidation, on the other hand, disclosed the presence of two components (see Fig. 1). The earlier eluted component was

* A factor of 2 should be included in the calculation of this value in ref. 13 to take account of both phosphate sites in I.

** A GLC method for the determination of Menadione has been reported[15].

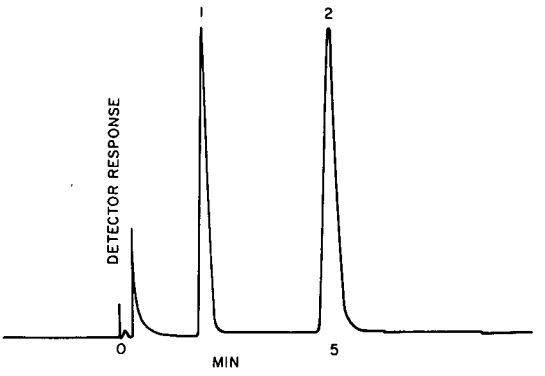


Fig. 1. GLC of the quinone fraction resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide. Column conditions are given in *Experimental*; temperature-programmed analysis: initial temperature 145°, 5°/min.

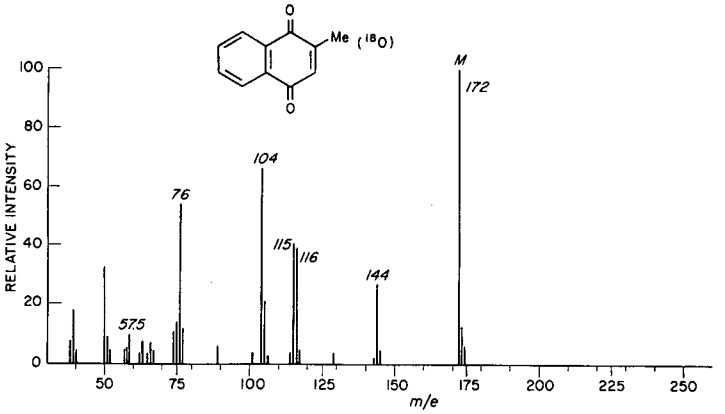


Fig. 2. Mass spectrum of the 2-methyl-1,4-naphthoquinone resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide.

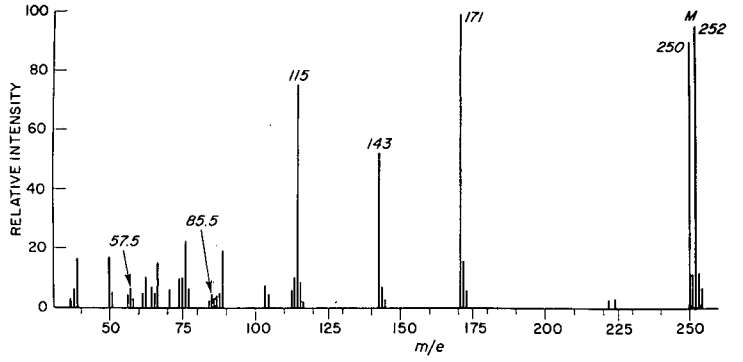


Fig. 3. Mass spectrum of the 3-bromo-2-methyl-1,4-naphthoquinone resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide.

identified by retention time and mass spectrum (see Fig. 2) as the expected Menadione (II). The mass spectrum of the later eluted component is presented in Fig. 3. This compound is clearly mono-bromo-Menadione—note the characteristic bromine isotope cluster for the molecular ion (*m/e* 250/252). NMR data on the mixture strongly suggest that the bromine atom is at the 3-position. The key NMR features leading to the structural assignment for the contaminant were the low areas of the $CH_3C = CH$ protons relative to the aromatic resonances and the appearance of a new methyl singlet at 7.62 τ. The two methyl peaks had a combined relative area of three protons. The absence of splitting in the 7.62 τ signal plus the downfield shift compared to that in Menadione are both diagnostic for a substituent at C-3. The significance of the production of this compound during the bromine oxidation of Synkavit is presently unclear*, although participation of such a species has previously been intimated[16].

The data obtained from the mass spectra indicated that II and P_i contain 2.9 and 5.2% excess ^{18}O, respectively (see *Experimental*). This would suggest that the oxidation in 17% enriched ^{18}O-dimethylformamide proceeded via III and IV to the extent of 31% and 17%, respectively. The former value (from P_i, as the tri-TMSi derivative) is seen to be consistent with, although greater than that noted previously[13]. The presently determined value (17%) for the second route (value obtained from 2-methyl-1,4-naphthoquinone uncontaminated with the bromination product) is also greater than the original value of 11% (ref. 13), perhaps because of the large amount of 3-bromo-2-methyl-1,4-naphthoquinone (V) present in the sample when it was investigated by the classical MS (conversion to CO_2) technique.

A disadvantage of the indirect (CO_2) approach for determination of ^{18}O (or ^{13}C) isotope content is that it does not allow differentiation between CO_2 from the compound of interest and that from possible sample contaminants. There is also the possibility of the introduction of impurities during combustion, or of incomplete combustion[17]. Although this method is capable of yielding isotope ratios with great accuracy[18], MS on the actual compound of interest, while producing less accurate data[18], does not suffer from the above disadvantages. The latter approach also allows the determination of isotope content on the molecular ion or appropriate fragment ion[19], providing a contaminant does not produce interfering ions. Of course, fractional sublimation of the components of a mixture may occur during sample evaporation into the ion source even with closely related compounds[20], and thus a contaminant will not necessarily volatilize with the compound of interest. However, the great separating power of GLC, resulting from the judicious choice of stationary phase, when combined with MS results in a superior technique for indicating non-homogeneity of sample and yielding isotope data on each component of a mixture on the microgram scale[11,12]. The potential of the method in chemical and biological studies employing limited amounts of partially purified compounds is obvious.

We wish to thank Mrs. VIRGINIA GRUBER for preparation of the ^{18}O-enriched phosphoric acid from P_i, Mr. JACK L. SMITH for assistance with the mass spectro-

* Calculations based on the signals at *m/e* 171 and 173 (M–Br) in the mass spectrum of bromo-Menadione suggest that this compound contains almost the same percent excess ^{18}O as the ^{18}O-enriched Menadione.

metric determinations, and Dr. JOHN L. BECK for his critical suggestions concerning the manuscript.

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1 R. RYHAGE, *Anal. Chem.*, 36 (1964) 759.
2 C. J. W. BROOKS, *Process Biochem.*, 2 (1967) 27.
3 P. CAPELLA AND C. M. ZORZUT, *Anal. Chem.*, 40 (1968) 1458.
4 E. C. HORNING, C. J. W. BROOKS AND W. J. A. VANDENHEUVEL, *Advan. Lipid Res.*, 6 (1968) 273.
5 J. A. MCCLOSKEY, *Methods Enzymol.*, XIV (1969) 382.
6 C.-G. HAMMAR, B. HOLMSTEDT AND R. KITZ, *J. Chromatogr.*, 49 (1970) 402.
7 W. J. A. VANDENHEUVEL, J. L. SMITH, I. PUTTER AND J. S. COHEN, *J. Chromatogr.*, 50 (1970) 405.
8 M. KURAŠ AND S. HÁLA, *J. Chromatogr.*, 51 (1970) 45.
9 H. SHAFER, W. J. A. VANDENHEUVEL, R. ORMOND, F. A. KUEHL AND F. J. WOLF, *J. Chromatogr.*, 52 (1970) 111.
10 W. J. A. VANDENHEUVEL, J. S. KELLER, H. VEENING AND B. R. WILLEFORD, *Anal. Letters*, 3 (1970) 279.
11 W. J. A. VANDENHEUVEL, J. L. SMITH AND J. S. COHEN, *J. Chromatogr. Sci.*, 8 (1970) 567.
12 M. ZINBO AND W. R. SHERMAN, *J. Amer. Chem. Soc.*, 92 (1970) 2105.
13 J. S. COHEN AND A. LAPIDOT, *J. Chem. Soc. (C)*, (1967) 1210.
14 W. C. BUTTS, *Anal. Letters*, 3 (1970) 29.
15 A. J. SHEPPARD AND W. C. HUBBARD, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 1093.
16 G. M. BLACKBURN AND J. S. COHEN, in M. GRAYSON AND M. GRIFFITH, (Editors), *Topics in Phosphorus Chemistry*, Vol. 6, New York, 1969, p. 222.
17 P. H. ABELSON AND T. C. HOERING, *Proc. Nat. Acad. Sci. U.S.*, 47 (1961) 623.
18 R. D. CRAIG, in R. I. REED (Editor), *Modern Aspects of Mass Spectrometry*, Plenum Press, New York, 1968, p. 29.
19 R. G. COOKS AND S. L. BERNASEK, *J. Amer. Chem. Soc.*, 92 (1970) 2129.
20 J. R. MAJOR AND R. PERRY, *J. Chem. Soc. (A)*, (1970) 822.

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CHROM. 5331

A method for the gas chromatographic determination of butyrophenones

Haloperidol and trifluperidol are potent and specific neuroleptics belonging to the butyrophenone series whose chemical structures are illustrated in Fig. 1.

The pharmacology of the butyrophenones has been extensively reviewed[1-4] and the clinical use of these compounds is continuously increasing. However, there

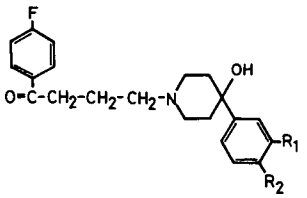

Fig. 1. Chemical structures of the butyrophenones: haloperidol: $R_1 = H$; $R_2 = Cl$; trifluperidol: $R_1 = CF_3$; $R_2 = H$.

are relatively few publications concerning the distribution and excretion of these drugs and their metabolites[5-9]. Tissue distribution of butyrophenones in animals has usually been studied by radioisotope techniques[10-12].

Although these techniques are very sensitive they cannot always answer the question whether the radioactivity found is wholly due to the administered labelled drug or to its metabolites.

In order to overcome the limitation of the above mentioned methods a simpler and more rapid procedure for the analysis of plasma and tissue levels of butyrophenones has been developed. The gas chromatographic (GC) method described here takes advantage of the presence of halogens in the chemical structure of butyrophenones which permits the use of an electron capture detector. The method described gives an excellent resolution and allows a quantitative estimation of haloperidol and trifluperidol intact.

Experimental

Reagents. All reagents must be of reagent grade purity. All inorganic reagents were made up in triply distilled water. 1 *M* KH_2PO_4 buffer, pH 7.2; diethyl ether: analytical reagent grade ether, containing not more than 0.00005% peroxides, was used. The bottle must be opened on the day of the extraction.

Extraction from blood. 2 ml of whole blood, 2 ml of buffer, 4 ml of H_2O and 10 ml of diethyl ether are placed in a glass-stoppered centrifuge tube. The tubes are shaken on a reciprocating shaker for 10 min and then centrifuged at 0° for 5 min. The ether phase is transferred to another glass-stoppered centrifuge tube. The water phase is re-extracted with 10 ml of ether and the ether extracts are combined.

5 ml of 6 *N* HCl are added to the ether extracts and the tubes are shaken for 10 min and then centrifuged at 0° for 5 min. The ether phase is discarded. The acidic phase is adjusted to a basic pH (10) and then is re-extracted twice with 10 ml of diethyl ether. The combined ether extracts are evaporated to dryness at 45° in a water bath.

The residue is dissolved in a suitable volume of a solution of internal standard in acetone. A suitable aliquot, from 1 to 3 μl, is injected in the GC column.

Extraction from tissue. Tissues are homogenized in cold absolute ethanol (1:10, w/v). The homogenate is then centrifuged at 9,000 $\times$ *g* for 20 min. The supernatant is evaporated to dryness and the residue is dissolved in 5 ml of 6 *N* HCl and extracted five times with 10 ml of diethyl ether. The ether phases are discarded. The acidic phase is adjusted to a basic pH (10) and then the procedure is identical to that described for blood extraction.

Gas chromatographic conditions. The gas chromatograph used was Model G I (Carlo Erba, Milan) equipped with a Ni-63 electron capture detector (voltage 50 V). The stationary phase was 3% OV-17 on Gas-Chrom Q (100–120 mesh) packed into a 2 m glass column (I.D. 2 mm, O.D. 4 mm). The flow rate of the carrier gas (nitrogen) was 40 ml/min or 60 ml/min and the column temperature was 270 or 280°, respectively, for the analysis of trifluperidol or haloperidol.

TABLE I

RELATION BETWEEN PEAK AREA AND AMOUNT OF BUTYROPHENONES

Butyrophenone	*Amount (ng)*	*R*[a]
Haloperidol	2	1.352
	5	2.544
	7	3.333
	10	4.391
Trifluperidol	1	0.246
	2	0.410
	3	0.556
	5	0.895

[a] R = [butyrophenone area (cm^2)]/[0.2 ng internal standard area (cm^2)].

Quantitative analysis. For identification and calculations the internal standard technique was used. 2-N-Benzylamino-5-chlorobenzophenone was chosen as an internal standard because of its suitable retention time. The area of the peaks was calculated by measuring, in convenient units, the height and width of the peak at half height. Butyrophenones can be quantitated by GC when the relative peak area is used as an index of concentration, since a linear relationship exists between relative peak area and haloperidol or trifluperidol concentration in the range of 2 to 10 ng and 1 to 5 ng, respectively, as reported in Table I.

Typical gas chromatograms showing the separation of butyrophenones analyzed and internal standard are illustrated in Fig. 2. Preliminary studies of the partition coefficient of butyrophenones between water and diethyl ether have shown that these drugs are substantially quantitatively extracted from the aqueous media with this organic solvent. The recovery from water and from rat blood, brain and adipose tissue is reported in Table II. Blanks from extracts of rat blood or tissue do not show peaks that interfere with a biological application of the GC procedure.

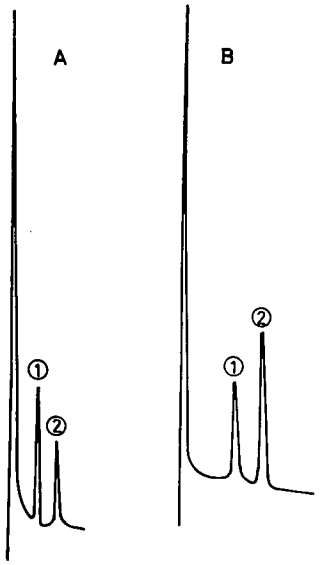

Fig. 2. Gas chromatographic separation of a mixture of: (A) internal standard (1) and haloperidol (2); (B) trifluperidol (1) and internal standard (2).

TABLE II

RECOVERY STUDIES

Compound	*% Recovery ± S.E. from*			
	Water	*Rat blood*	*Rat brain*	*Rat adipose tissue*
Haloperidol	90 ± 2	72 ± 2	73 ± 3	70 ± 1
Trifluperidol	92 ± 3	82 ± 2	83 ± 1	90 ± 1

The method described may be extended to the analysis of other members of the butyrophenone chemical series. The high sensitivity of the method suggests its application to the determination of the level at butyrophenones in the blood of animals and humans.

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1 T. BAN, *Psychopharmacology*, Williams and Wilkins, Baltimore, 1969, p. 238.
2 P. A. J. JANSSEN, in P. B. BRADLEY, F. FLUGEL AND P. H. HOCH (Editors), *Neuropsychopharmacology, Proc. 3rd Meeting of the Collegium Internationale Neuropsychopharmacologicum*, Elsevier, New York, 1964, p. 331.
3 P. A. J. JANSSEN, *Int. Rev. Neurobiol.*, 8 (1965) 221.
4 P. A. J. JANSSEN, C. J. NIEMEGEERS, K. H. L. SCHELLEKENS AND F. M. LENAERTS, *Arzneimittelforsch.*, 17 (1967) 841.
5 G. A. BRAUN, C. F. JR. KADE AND E. L. ROSCOE, *Int. J. Neuropsychiat.*, 3, suppl. 1, (1967) 22.
6 W. SOUDIJN, I. VAN WIJNGAARDEN AND F. ALLEWIJN, *Eur. J. Pharmacol.*, 1 (1967) 47.
7 G. A. BRAUN, G. I. POOS AND W. SOUDIJN, *Eur. J. Pharmacol.*, 1 (1967) 58.
8 P. A. J. JANSSEN, W. SOUDIJN, I. VAN WIJNGAARDEN AND A. DRESSE, *Arzneimittelforsch.*, 18 (1968) 282.
9 G. SCARLATO AND G. PELLEGRINI, *Psychopharmacologia*, 11 (1967) 165.
10 P. A. J. JANSSEN AND F. T. N. ALLEWIJN, *Arzneimittelforsch.*, 19 (1969) 199.
11 R. VERTURA AND A. FURLANI, *Eur. J. Pharmacol.*, 3 (1968) 347.
12 P. A. J. JANSSEN ADN F. T. N. ALLEWIJN, *Arzneimittelforsch.*, 18 (1968) 279.

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Amino acid analysis: The reduction of ninhydrin reagent with titanous chloride

Reduced ninhydrin (hydrindantin) can be added to the reagent mixture used with amino acid analyzers for colour development[1] or it can be formed in the reagent mixture by the addition of stannous chloride[2,3] (reduction can also be effected with cyanide[4] or ascorbic acid[5,6]). When the hydrindantin is added directly to the reagent mixture the disadvantages are that great care must be exercised to prevent air oxidation taking place during transfer to the analyzer reservoir[1] and a delay of 12 h is recommended before the mixture is ready for use. When the hydrindantin is formed by the addition of stannous chloride, precipitation of tin salts eventually occurs in the reagent reservoir and flow lines of the analyzer. Where a piston ninhydrin pump is used to supply ninhydrin mixture this salt deposit could give rise to variations in the pumping rate. Periodic flushing of the system with water will remove this precipitate.

It has been found that commercially available titanous chloride solutions can be used successfully and more conveniently in place of stannous chloride in the preparation of reduced ninhydrin reagent for use in automatic amino acid analysis. Furthermore, no precipitate has been observed to occur in the reagent mixture upon standing.

Titanous chloride solution L.R. (15% w/v) was supplied by Hopkin and Williams Ltd., Essex, Great Britain, and contained 14.78% w/v of $TiCl_3$ (gravimetric determination using Cupferron). Ninhydrin and methyl cellosolve, 'Sequanal' grade, were supplied by the Pierce Chemical Co., Rockford, Ill., U.S.A. 'Analar' sodium acetate and potassium acetate were supplied by B.D.H., Poole, Dorset, Great Britain. 'Univar' stannous chloride was obtained from Ajax Chemicals Ltd., Sydney, Australia. The amino acid analyses were carried out on a Technicon Auto-Analyzer.

The ninhydrin reagent mixture was prepared according to SPACKMAN *et al.*[2], except that a mixed sodium/potassium acetate buffer[7] and a lower concentration of ninhydrin were used, the reduction being effected with either stannous chloride or titanous chloride. The mixture used contained 60 g of ninhydrin dissolved in a 3:1 mixture of methyl cellosolve and acetate buffer. The final volume of the reagent was 4 l and the ninhydrin was reduced with either 7.5 ml of titanous chloride solution or 1.2 g of stannous chloride. The reagent was kept under nitrogen.

When the titanous chloride is added the reagent mixture immediately changes colour from yellow to red (which fades to brown) and after an interval of 30 min is ready for use. It is worth noting the convenience of a pipette addition of titanous chloride solution over the weighing out of the stannous chloride in the preparation of the reagent mixture. Although titanous chloride, like stannous chloride, is susceptible to air oxidation[8] no significant difference could be detected in the colour development potential of a reagent mixture prepared utilizing titanous chloride solution recently acquired from the supplier or another sample, estimated to be thirteen years old, from the same supplier.

The amino acid analyses were obtained by using a two-column system. The short column contained Beckman M81 resin (height of resin, 6 cm) and the long

TABLE I

CALIBRATION CONSTANTS[a] OBTAINED WITH A SYNTHETIC AMINO ACID MIXTURE

Amino acid	*$SnCl_2$ reduction*	*Range*		*$TiCl_3$ reduction*	*Range*	
		High	*Low*		*High*	*Low*
Lys	68.5	69.0	68.0	73.6	76.5	72.6
His	63.8	65.2	61.7	66.8	68.2	64.6
Arg	67.0	68.4	64.8	65.5	66.7	64.5
Asp	57.0	58.5	55.5	53.7	55.0	51.4
Thr	61.0	62.5	60.5	59.0	61.2	57.0
Ser	63.0	65.0	61.0	61.6	63.0	60.0
Glu	65.0	66.0	63.0	64.2	65.0	63.4
Pro	15.6	16.4	14.6	15.9	16.8	15.0
Gly	63.5	66.0	62.5	63.1	66.7	61.0
Ala	65.2	66.5	64.5	64.7	66.3	63.0
½Cys	33.5	33.8	33.2	32.7	33.3	31.9
Val	64.1	66.0	62.0	63.0	64.8	62.7
Met	64.7	66.5	63.1	65.5	66.7	64.3
Ile	63.2	67.0	62.0	63.0	65.0	62.4
Leu	64.5	67.0	64.0	63.0	65.0	60.5
Tyr	62.5	64.0	61.5	62.0	62.8	61.4
Phe	62.3	63.7	60.6	61.4	62.2	60.8

[a] Average of five analyses.

column contained Technicon Chromobead Type A resin (height of resin, 57 cm). Buffer was pumped at 60 ml/h and the AutoAnalyzer was modified to allow the buffer to go directly into the mixing coil prior to the reaction bath coil (by-passing the proportioning pump). Nitrogen was pumped at approximately 50 ml/h and the ninhydrin at 60 ml/h. A double glass coil approximately 75 ft. long was used in the reaction bath and the reaction mixture took 15 min to go through the coil. The flow cells had a 15-mm light path and the absorbance of the reaction mixture was monitored at 570 nm and 440 nm.

The amino acid calibration mixture used was prepared in this laboratory and was applied to the ion-exchange columns at a concentration of 0.1 μmoles of each amino acid per ml. The volume applied was 1 ml. The data in Table I were obtained utilizing the two methods of reducing ninhydrin described above. The figures given are the average of five analyses and were calculated by dividing the $H \times W$ factor[2] by the concentration of the amino acid in the sample. These values are referred to as calibration constants.

The results presented in Table II relate to the stability of the ninhydrin reagent mixture prepared with titanous chloride. The calibration constants in column No. 1 were obtained 24 h after reagent preparations and those in column No. 2 28 days later (reagent kept under nitrogen). From the constants shown in Table II it can be seen that no deterioration in the colour development potential of the reagent can be detected.

To determine the working range of the titanous chloride-reduced ninhydrin reagent, the amino acid arginine was selected as being the most useful for this purpose; the arginine peak is low and broad on the chromatogram while most of the other amino acids, when present in excess of 0.1 μmoles, give peak heights too large to be estimated accurately on the logarithmic scale of the recorder.

TABLE II

CALIBRATION CONSTANTS OBTAINED OVER A PERIOD OF 28 DAYS WITH TITANOUS CHLORIDE AS THE NINHYDRIN REDUCING AGENT

Amino acid	*No. 1* (*24 h*)	*No. 2* (*28 days*)	*Amino acid*	*No. 1* (*24 h*)	*No. 2* (*28 days*)
Lys	76.5	72.2	Ala	64.8	65.3
His	66.0	64.6	½Cys	33.3	32.8
Arg	65.6	65.8	Val	60.2	64.8
Asp	51.4	54.0	Met	65.8	64.3
Thr	57.3	60.8	Ile	61.6	62.1
Ser	61.0	63.0	Leu	62.4	63.5
Glu	63.4	65.0	Tyr	61.4	61.5
Pro	15.5	16.1	Phe	60.8	61.0
Gly	63.0	62.7			

Mixtures of amino acids, containing (a) 0.1 μmoles, of each amino acid, (b) 0.2 μmoles, (c) 0.3 μmoles, and (d) 0.4 μmoles were applied to the short column in consecutive analyses. The $H \times W$ factor[2] for arginine was calculated in each case and the calibration constants were found to be (a) 66.0, (b) 66.1, (c) 67.1, and (d) 62.5. These results indicate that over the most useful and accurate range of the recorder peak print-out, a linear colour development takes place and that the potential of the reagent mixture for developing colour with amino acids is far in excess of that which would be required to maintain optimum conditions for quantitative analyses.

Whenever the titanous chloride reducing agent was replaced by stannous chloride (and *vice versa*) on consecutive analyses no significant change was required in the colorimeter potentiometer settings to maintain the correct locations for the recorder baseline print-out.

The above results show that, as a reducing agent for ninhydrin, titanous chloride is superior to stannous chloride in ease of mixture preparation and in the absence of salt precipitation within the flow lines of the analyzer. Satisfactory results are also given in colorimetric analysis.

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1 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
2 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
3 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1954) 367.
4 E. SCHWERDTFEGER, *J. Chromatogr.*, 7 (1962) 418.
5 R. ABDEHALDEN, *Z. Physiol. Chem.*, 252 (1938) 81.
6 E. S. WEST AND R. E. RINEHART. *J. Biol. Chem.*, 146 (1942) 907.
7 D. EAKER, *Symposium on Evaluation of Novel Protein Products, Stockholm, Sweden, Sept., 1968.*
8 A. I. VOGEL, *Textbook of Quantitative Inorganic Analysis*, 3rd Ed., Longmans, London, 1962, p. 329.

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CHROM. 5360

Chromatography of cysteine and glutathione derivatives on a Dowex 1/Sephadex mixed-bed column

As reported previously[1], Dowex 1 X8 anion-exchange resin may be employed to separate many sulfinic and sulfonic acids which co-chromatograph on cation-exchange resins such as those normally employed in automatic amino acid analyzers. However, even on Dowex 1 X8, many important amino acids and peptides co-chromatograph and we had difficulty while trying to measure sulfinic and sulfonic acid derivatives of cysteine and glutathione. Cysteic acid had the same elution time as glutathione disulfide which was always present in our samples. It was thought that addition of Sephadex G-10 to the resin bed would retard the smaller molecules enough to make a separation possible.

Materials and methods

Most of the amino acids used were purchased from Calbiochem or Mann Research Laboratories. Analysis showed that the cysteine sulfinic acid was not a hydrate as indicated on the label. Preparation of some of the compounds has been described previously[1]. Glutathione sulfinic acid was prepared as described by CALAM AND WALEY[2]. It was purified by chromatography on a column of Dowex 1 X8, formate form, using a formic acid gradient[3]. The sulfinic acid was detected by the sodium iodoplatinate test. Glutathione sulfonic acid was made by oxidising glutathione with performic acid and purified as described in the literature[4]. A mixture containing cysteine and glutathione disulfides and trisulfides was prepared by the method of FLETCHER AND ROBSON[5,6] whereby the thiols were reacted with free sulfur dissolved in chloroform and ethanol. After evaporation of the solvent the residue was dissolved in water, filtered, and freeze dried.

The amino acid analyzer was a modified Technicon[1], model NC-1, and the standard 0.6 × 140 cm glass columns were used to contain the resin. The mixed-bed column was prepared by mixing Dowex 1 X8 (minus 400 mesh) anion-exchange resin (40 ml) previously converted to chloroacetate form, and Sephadex G-10 (25 ml). A slurry of the mixture in water was poured into the glass column and packed as rapidly as possible by applying air pressure in order to prevent separation of the Dowex and the Sephadex. An "autograd" gradient elution device was used to produce a suitable gradient. Water containing BRIJ-35 (10 ml/l) and thiodiglycol (5 ml/l), and sodium monochloroacetate solution (1 *M*, pH 5.5) containing BRIJ-35 (10 ml/l) were used in the "autograd". Using seven chambers, with 100 ml in each, the volume (ml) of chloroacetate was increased gradually from chambers 1 to 7 as follows: 0, 5, 20, 40, 60, 80, 100.

Results and discussion

The elution of the sulfinic and sulfonic acids of cysteine and glutathione from Dowex 1 X8 at room temperature is illustrated in Fig. 1. As illustrated, the cysteic

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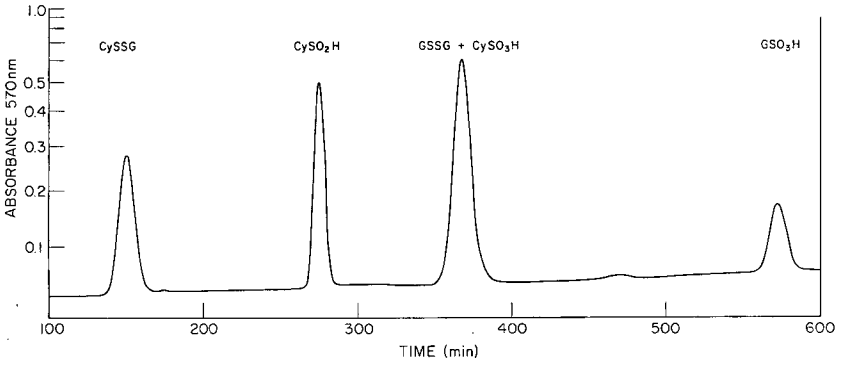

Fig. 1. Chromatography of disulfides and sulfinic and sulfonic acids on a column of Dowex 1 at room temperature and 30 ml/h buffer flow rate.

acid and glutathione disulfide peaks coincided and this was usually the case on Dowex 1 columns. Increasing the column temperature from room temperature to 45° and decreasing the buffer flow rate from 30 to 20 ml/h produced a partial separation but the peaks became broad and the elution times increased to an undesirable extent. Columns with mixed beds of Dowex 1 X8 and Sephadex G-10 were prepared and tested with the same mixture of amino acids. The proportion of Sephadex in the column was increased until a good separation was obtained. Good chromatograms were achieved using a buffer flow rate of 20 ml/h and a column temperature of 45°. Under these conditions the run was still reasonably fast due to the reduction in the amount of anion-exchange resin in the column. Fig. 2 shows the elution pattern obtained with a column bed consisting of 8 parts Dowex 1 X8 and 5 parts Sephadex G-10. The amino acid mixture contained *ca.* 0.25 μequiv. of each amino acid except for glutathione sulfonic acid which was half strength. Several of our standard amino acids were chromatographed on the Dowex 1/Sephadex column and Table I compares the elution times with a Dowex 1 column of the same dimensions. The yields shown in the table are relative to glutamic acid; the actual yields may vary de-

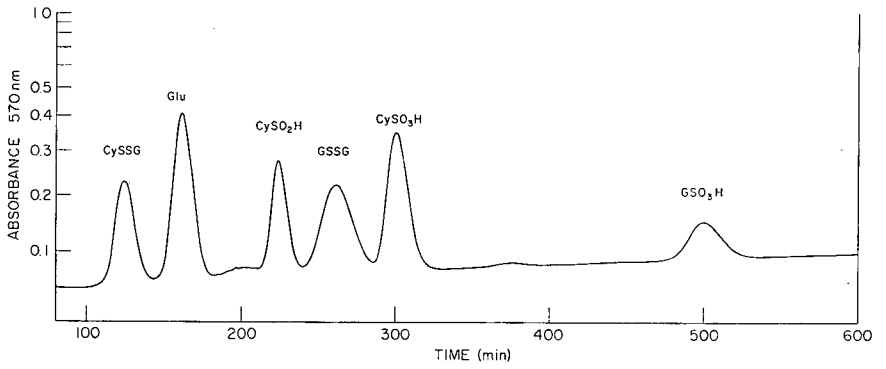

Fig. 2. Chromatography of disulfides and sulfinic and sulfonic acids on a mixed-bed column of Dowex 1 and Sephadex G-10 at 45° and 20 ml/h buffer flow rate.

TABLE I

ELUTION OF ACIDS FROM DOWEX 1/SEPHADEX AT 45° AND 20 ML/H AND FROM DOWEX 1 AT ROOM TEMPERATURE AND 30 ML/H

Compound	*Abbreviation*	*Elution time (min)*		*Yield (hw/0.25 μ equiv.)*
		Dowex/ Sephadex	*Dowex 1*	
Cystine	CySSCy	35	30	7.5
Cysteine-glutathione disulfide	CySSG	125	150	9.5
Glutamic acid	Glu	160	230	13.5
Cysteine sulfinic acid	$CySO_2H$	225	275	10.9
Glutathione disulfide	GSSG	260	365	10
Cysteic acid	$CySO_3H$	300	365	10.5
Glutathione sulfinic acid[a]	GSO_2H	375	480	10
Glutathione sulfonic acid	GSO_3H	500	575	10.0
Alanine thiosulfonic acid	$CySO_2SH$	505	615	—
Alanine thiosulfuric acid	$CySSO_3H$	540	660	8.0

[a] A trace of GSO_2H can be seen in the runs illustrated in Figs. 1 and 2.

pending on the system used, flow rates, etc. The glutathione sulfinic acid was impure but it was assumed to have a color yield similar to the sulfonic acid by analogy with other sulfinic and sulfonic acids. The color yield of glutathione sulfonic acid was checked by hydrolyzing a sample and measuring the yields of glycine, glutamic and cysteic acids.

The mixed-bed column had the good elution properties of the ion-exchange resin used combined with the molecular sieve features of Sephadex G-10. After a period of time the column bed appeared to pack down and the elution times increased but the elution pattern did not change significantly. It may be necessary to repour the column after several months or to fill it with fresh Dowex 1/Sephadex mixture. Similar separations should be possible using anion-exchange Sephadex but these materials are not available at present in a molecular exclusion range corresponding to Sephadex G-10. One anion-exchange Sephadex was tested, QAE-Sephadex A-25,

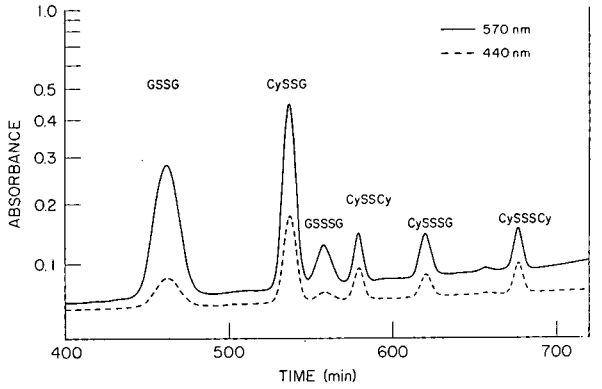

Fig. 3. Chromatography of disulfides and trisulfides of cysteine and glutathione on "Chromobeads" at 60° and 30 ml/h buffer flow rate.

but it was unsuccessful and tended to shrink as the ionic strength of the eluant was increased.

While working on glutathione derivatives it became necessary to determine the elution times of glutathione trisulfide and the mixed trisulfide of cysteine and glutathione. A mixture of these compounds, together with the corresponding disulfides, was chromatographed on "Chromobeads" type A. This column was operated at 60° with the gradient solution described previously[6]. The elution pattern is illustrated in Fig. 3. As before, the ratio of the absorption at 570 nm to the absorption at 440 nm was useful for identifying derivatives of cystine. This mixture of disulfides and trisulfides was also resolved successfully on a shorter column (0.6 × 75 cm) of chromobeads type C2 with a buffer flow rate of 30 ml/h. The order of elution of the amino acids was unchanged but the elution times were reduced to: GSSG, 235; CySSG, 275; GSSSG, 285; CySSCy, 295; CySSSG, 320; CySSSCy, 355. The disulfides were formed in greater yield than the trisulfides whereas the opposite effect was observed when the trisulfides of cysteine and penicillamine were made by the same method.

In conclusion, Sephadex can be added to an ion-exchange resin in order to improve the separation where compounds of significantly different molecular weights are involved. It should be possible to apply this technique to the separation of other substances which differ sufficiently in ionic properties and molecular weights.

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1 J. W. PURDIE, J. P. FARANT AND R. A. GRAVELLE, *J. Chromatogr.*, 23 (1966) 242.
2 D. H. CALAM AND S. G. WALEY, *Biochem. J.*, 85 (1962) 418.
3 C. DAVIES, R. D. HARTLEY AND G. J. LAWSON, *J. Chromatogr.*, 18 (1965) 47.
4 W. O. KERMACK AND N. A. MATHESON, *Biochem. J.*, 65 (1957) 45.
5 J. C. FLETCHER AND A. ROBSON, *Biochem. J.*, 87 (1963) 553.
6 J. W. PURDIE, R. A. GRAVELLE AND D. E. HANAFI, *J. Chromatogr.*, 38 (1968) 346.

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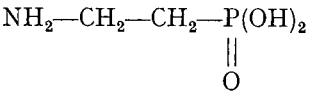
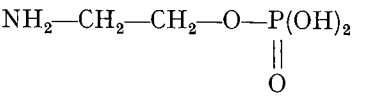
CHROM. 5343

The separation of ciliatine and phosphorylethanolamine

In the last decade, ciliatine or 2-aminoethylphosphonic acid (I) has been detected in the lipid and protein fraction of a great variety of invertebrates[1]. Its structure and its chromatographic properties are similar to phosphorylethanolamine (II) which has also been found in the lipids of some invertebrates[2,3].



(I)



(II)

Ciliatine may be readily distinguished from phosphorylethanolamine by its resistance towards drastic hydrolysis with hydrochloric acid, since phosphorylethanolamine is completely hydrolysed into ethanolamine and phosphoric acid on prolonged hydrolysis whereas ciliatine remains unchanged. It is still important however to have a genuine separation procedure which allows the identification and determination of ciliatine and phosphorylethanolamine in a mixture of both these compounds.

Recently NEUZIL *et al.*[4] published a method for the separation of ciliatine and phosphorylethanolamine on ion-exchange paper; this method is however unsuitable for quantitative work.

The present paper describes the separation of these two compounds on a column of the ion-exchange resin Amberlite CG 120 at a pH value of 2.50 and a temperature of 50°. After separation the compounds are estimated by means of their reaction with ninhydrin.

Experimental

The ion-exchange resin, Amberlite CG 120 type 2, was packed to a height of 120 cm in a chromatography column (125 × 0.9 cm) which was jacketed in order to permit temperature control with water. The eluting buffer was prepared by dissolving 105 g of citric acid, 41 g of sodium hydroxide and 52 ml of concentrated hydrochloric acid in 5 l of water; the pH of the buffer was adjusted to the required value with dilute hydrochloric acid or sodium hydroxide.

Ciliatine was obtained from Prof. A. F. ISBELL of Texas A. and M. University and a solution containing 580 μg ciliatine per ml of citrate buffer was prepared. Phosphorylethanolamine was purchased from L. Light and Co. Ltd., Colnbrook, Great Britain and a solution containing 248 μg of phosphorylethanolamine per ml of citrate buffer was prepared.

For the separation experiments 0.5 ml of each of the solutions *i.e.* 290 μg ciliatine and 124 μg phosphorylethanolamine were mixed and carefully placed on the column. The substances were eluted from the column with the citrate buffer and 1-ml fractions were collected with an automatic fraction collector. Ciliatine and phosphorylethanolamine were assayed in the tubes by reaction with ninhydrin in a boiling water bath according to MOORE AND STEIN[5]. After dilution with 10 ml of water the absorbance was read at 565 nm and plotted against tube number. Quanti-

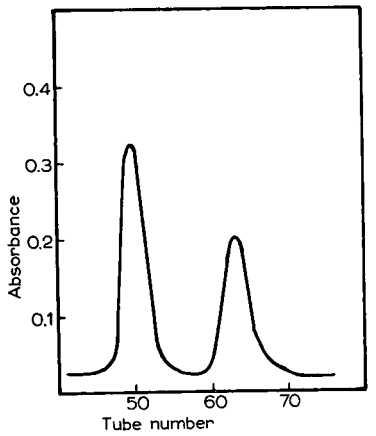


Fig. 1. Separation of 124 μg phosphorylethanolamine and 290 μg ciliatine on the ion-exchange resin Amberlite CG 120 at 50° with a citrate buffer at pH 2.50.

tative estimation of ciliatine in lipid hydrolysates was achieved by comparing the peak area with that obtained from the standard solution.

The extraction and the hydrolysis of lipids have been described elsewhere[6].

Results and discussion

Fig. 1 shows the separation of 124 μg of phosphorylethanolamine and 290 μg of ciliatine on the ion-exchange resin at a pH value of 2.50. Tests with phosphorylethanolamine or ciliatine alone indicated that the first peak is due to phosphorylethanolamine and the second due to ciliatine. Previous work[6] had shown that phosphorylethanolamine and ciliatine could only be partially separated on the ion-exchange resin using a citrate buffer of pH 3.25 (*cf.* ref. 2); at this pH the compounds form a double peak, phosphorylethanolamine having a maximum at tube number 48 and ciliatine at tube number 52. By lowering the pH of the eluting buffer to 2.50 the ciliatine peak shifted considerably, its maximum now being at tube 63, whereas the shift of the phosphorylethanolamine peak was negligible; thus the separation at pH 2.50 was complete as is clearly shown in Fig. 1. This characteristic shift may therefore be regarded—as was done in the present paper—as additional evidence for the presence of ciliatine in hydrolysates.

By means of the above-mentioned procedure we were able to detect and

TABLE I

CILIATINE IN THE LIPIDS OF A MARINE CRAB (*Cyclograpsus punctatus*) AND THE GARDEN SNAIL (*Helix aspersa*)

Source	*Percentage ciliatine in lipids*
Marine crab body	0.10
Marine crab viscera	0.08
Snail body	0.34
Snail viscera	0.19

estimate ciliatine in the lipids of a marine crab (*Cyclograpsus punctatus*) and the garden snail (*Helix aspersa*). The results of this work are shown in Table I and indicate that the body and viscera lipids of the crab and the snail contain small amounts of ciliatine.

I wish to thank JOHN MALIBO and PETER KULEHILE for assistance in collecting and analysing the snails.

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1 J. S. KITTREDGE AND E. ROBERTS, *Science*, 164 (1969) 37.
2 T. HORI, I. ARAKAWA AND M. SUGITA, *J. Biochem. (Tokyo)*, 62 (1967) 67.
3 T. HORI, M. SUGITA AND I. ARAKAWA, *Biochim. Biophys. Acta*, 152 (1968) 211.
4 E. NEUZIL, H. JENSEN AND J. LE POGAM, *J. Chromatogr.*, 39 (1969) 238.
5 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
6 A. J. DE KONING, *Biochim. Biophys. Acta*, 202 (1970) 187.

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CHROM. 5341

Rapid and sensitive measurement of 7-methylguanosine and N^6-isopentenyl derivatives of adenosine by cation-exchange chromatography

Because pure nucleic acids are generally available only in relatively small quantities, the nondestructive analysis of nucleic acid derivatives by UV spectrophotometry of chromatographic effluents continues to offer several advantages. The sensitivity of the procedure is dependent largely upon the construction of the columns and monitoring units. For example, it is possible to analyze 0.3 μg of nucleosides or bases ($\sim$ 1 nmole) and to detect as little as 0.015 μg ($\sim$ 0.05 nmole) by cation-exchange chromatography[1,2]. This analytical sensitivity compares favorably with both gas chromatography[3–6], radioactive measurements[7], and biological assays. One can monitor the effluent at a number of wavelengths so that spectral data as well as peak position are obtained. In addition, when volatile solvents are used the isolated material is readily recoverable in an unmodified form for further chemical, mass spectrographic, or biological assay.

One disadvantage of cation-exchange chromatography of the minor nucleosides and bases of RNA has been that strongly positively charged and highly hydrophobic substances are not readily eluted with totally aqueous solvents. For example, in the system described by UZIEL *et al.*[1], the compound 2-methylthio-N^6-isopentenyladenosine is eluted at about 64 h elution time, compared to about 3 h for most of the known nucleosides. The band is broad and not readily detectable. By incorporating ethanol into the solvent, we can now separate within 2.5 h a group of nucleosides characterized by high positive charge and/or a strong lyophobic substituent*.

Materials and methods

The instrumentation and chemical standards have been described previously[1]. 2-Methylthio-N^6-isopentenyladenosine was a gift of Dr. NELSON LEONARD. N^6-Isopentenyladenosine was a gift from Dr. R. H. HALL. *E coli* $tRNA_1^{Tyr}$ was prepared in our laboratory by chromatography of crude tRNA on Sephadex A-50 (ref. 8) followed by chromatography with the RP-4 system of KELMERS *et al.*[9]. This preparation was more than 90% homogeneous as judged by tyrosine acceptance (1550 pmole per A_{260} unit)**. Hydrolysis of this tRNA prior to analysis was done by a modification of the procedure previously described[1]. The tRNA was initially treated with RNase T_1 (0.1 mg/ml final concentration in 0.02 *M* ammonium acetate, pH 7.2) for 30 min at 37°.

The sequential use of RNase T_1 and a combination of venom diesterase and alkaline phosphatase[1] totally degrades tRNA to nucleosides. Although the combination of venom diesterase and phosphatase will, in theory, hydrolyze any tRNA, we find some samples to be refractory to only this treatment. The prior RNase T_1 step has always led to total hydrolysis. Any unhydrolyzed or cyclic phosphates appear at the breakthrough position.

* Similar decreased retention volumes and peak widths have been observed by R. P. SINGHAL and W. E. COHN on anion-exchange chromatography.

** One A_{260} unit is the amount of RNA that gives an absorbance of 1.0 when it is dissolved in 1 ml of water and read in a path length of 1 cm at 260 nm.

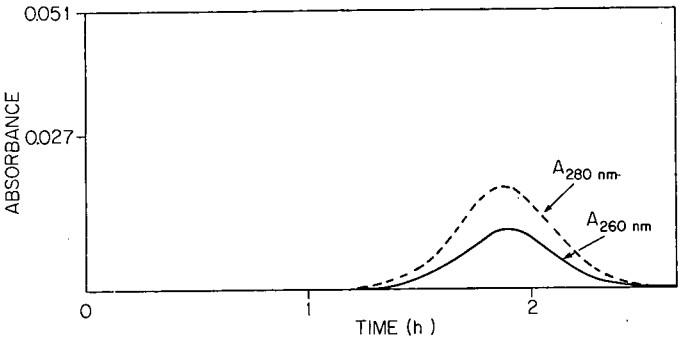

Fig. 1. Chromatography of 2-methylthio-N^6-isopentenyladenosine on a column of cation-exchange resin (Bio Rad A-6) with the dimensions 13 × 0.5 cm. The solvent had a final composition of 0.85 *M* ammonium acetate (pH 5.7) and 15% ethanol, and the temperature was maintained at 49°. A sample of 2.7 μg of compound was passed through the column at a flow rate of 0.29 ml/min.

Results and discussion

The simple inclusion of ethanol in the solvent described in our earlier paper caused the 2-methylthio-N^6-isopentenyladenosine to elute at about 16 h rather than the original 64 h[1]. We then increased the ionic strength and pH from 0.4 *M* and pH 4.65 to the values given in Fig. 1. The higher pH reduces the positive charge on groups ionizing near pH 4.7, the increased ionic strength reduces the retention volume due to ion exchange, and the ethanol increases the solubility of the compound(s) in the moving phase, again causing smaller retention volumes and narrower peak widths.

Fig. 1 illustrates the chromatography of 2-methylthio-N^6-isopentenyladenosine

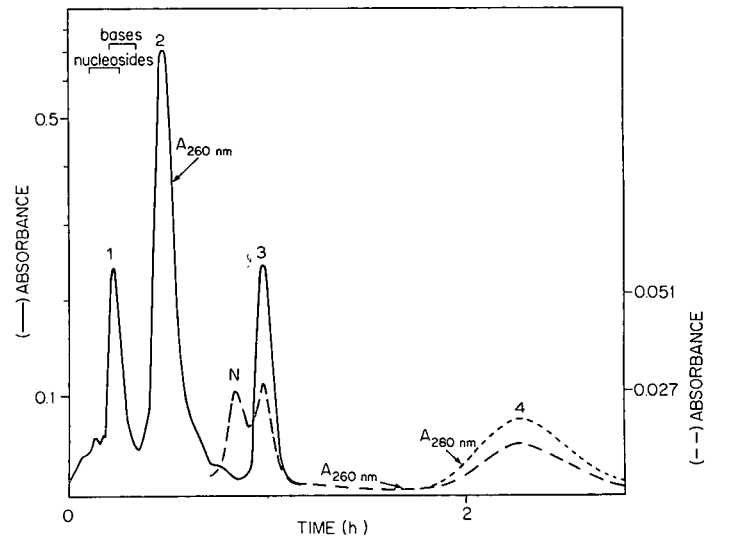

Fig. 2. The column and solvent described in Fig. 1 was used to obtain the positions of the usual four nucleosides, four bases (brackets), adenosine (1), N^6-isopentenyladenosine (2), 7-methylguanosine (3), and an enzymatic hydrolysate of *E. coli* $tRNA_1^{Tyr}$ (– – – —) which contained an unknown compound N as well as 7-methylguanosine and 2.6 μg 2-methylthio-N^6-isopentenyladenosine (4). The flow rate was 0.25 ml/min.

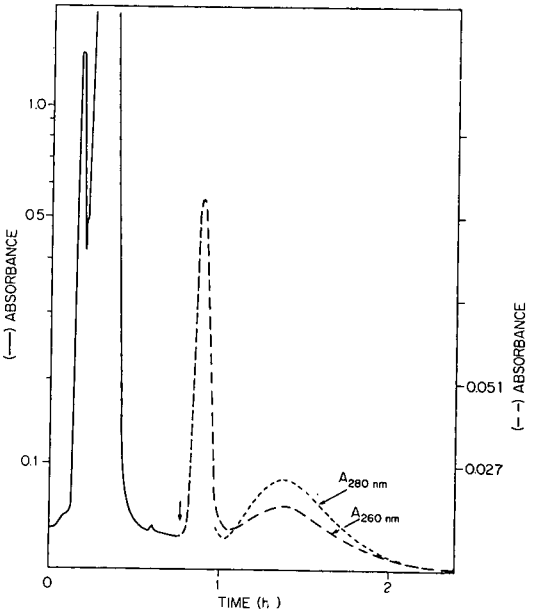

Fig. 3. Increasing the ethanol concentration resulted in an earlier elution of 2-methylthio-N^6-isopentenyladenosine. The column described in Fig. 1 was used and the solvent composition was 0.8 *M* sodium acetate and 20% ethanol. The assay was complete in about 2 h.

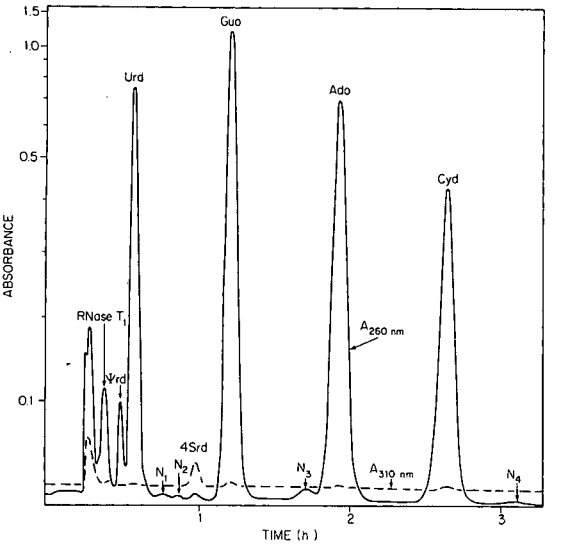

Fig. 4. A column, 23 × 0.6 cm, of cation exchanger[1] was used to separate the nucleoside obtained from mixed *E. coli* B tRNA. About 3 nmoles of tRNA were analyzed. Ψrd = pseudo-uredine; Urd = uridine; 4 Srd = 4-thiouridine; Guo = guanosine; Ado = adenosine; Cyd = cytidine.

in 0.85 *M* ammonium acetate, pH 5.7 (15% ethanol v/v). The sample contained 2.7 μg of material, based upon the spectral data of BURROUGHS *et al.*[10]. One-fifth of this amount would be readily detected in this system. The sensitivity of this assay is comparable to the biological assay of N^6-substituted adenines using the chlorophyll preservation test[11] described in Table I. A reduction of the column diameter ($\times$ 1/2) and the volume in the flow cell can further increase the sensitivity fourfold.

Fig. 2, which is a composite of two chromatograms, shows the separation of adenosine, N^6-isopentenyladenosine, 7-methylguanosine, 2-methylthio-N^6-isopentenyladenosine, and an unknown compound N. The 7-methylguanosine was a synthetic standard. The dashed lines represent a hydrolysate of *E. coli* B $tRNA_1^{Tyr}$. We routinely scan at least two wavelengths to give supporting evidence for the identity of the compound. The yield of 2-methylthio-N^6-isopentenyladenosine was 1450 pmole/A_{260} indicating 0.94 moles/mole tRNA. The brackets at the front of the chromatogram show the elution positions for the usual nucleosides and bases.

Fig. 3 illustrates the effect of increasing the ethanol concentration to 20% and reducing the salt concentration to 0.8 *M* ammonium acetate. The sample was a hydrolysate of $\sim$ 16 nmole of *E. coli* B $tRNA^{Phe}$ (65% pure prepared by the Oak Ridge National Laboratory). The yield of 7-methylguanosine is low (see later paragraphs). For comparison, Fig. 4 shows a typical pattern of nucleosides obtained from mixed *E. coli* tRNA chromatographed in the usual way[1].

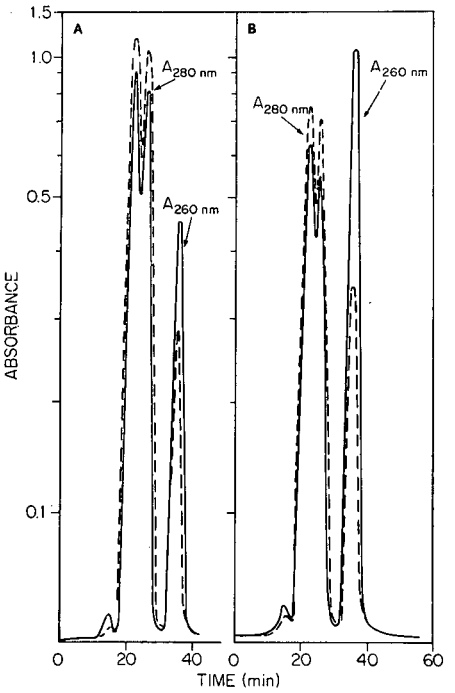

Fig. 5. Analyses done on the column described in Fig. 4. Seven nmoles of 7-methylguanosine were hydrolyzed at 80° in 0.3 *N* NaOH for 15 min (A) and 40 min (B). The A_{280}/A_{260} ratio was constant over the double peak near the breakthrough, and the loss of A_{260} from this double peak on prolonged treatment with alkali was recovered entirely in the increase of absorbance in peak 3. This suggests that all three compounds have the same extinction coefficient.

TABLE I

ASSAY FOR CHLOROPHYLL PRESERVATION BY ADDED ADENOSINE DERIVATIVES

Additions[a]	*M*[b]	*μg chlorophyll remaining*[c]	*Net chlorophyll remaining in leaf tips*[d]
Four days' incubation		19 ± 2	0
No incubation		40 ± 7	21
A-A-Ap	1×10^{-6}	20 ± 3	0
A-A-Gp	1.2×10^{-6}	20 ± 1	0
Kinetin	1×10^{-6}	36 ± 2	17
2-Methylthio-N^6-isopentenyladenine	0.5×10^{-6}	34 ± 6	15

[a] The assay consists of incubation of eight wheat leaf tips for four days in 0.05 *M* potassium phosphate buffer, pH 6.5, in the absence (lines 1 and 2) or presence of various nucleotide derivatives. All additions were hydrolyzed for 1 h at 100° in 1 *N* HCl and neutralized prior to addition to the assay medium (final volume 5 ml).

[b] Molarity of the substance in the assay medium (2.5 to 5 nmoles were used in the various experiments).

[c] Measured residual chlorophyll normalized to one wheat leaf tip.

[d] This value is obtained by subtracting the amount of chlorophyll remaining in the wheat leaf tips after four days' incubation in just the buffer.

The procedures we have described here give quantitative recovery of 2-methylthio-N^6-isopentenyladenosine, N^6-isopentenyladenosine, or 7-methylguanosine from the column chromatography. The overall low yields of 7-methylguanosine we have observed are due mainly to the conditions for preparation of the nucleosides. For example, alkaline hydrolysis followed by phosphatase yields three new components from the original 7-methylguanosine on cation-exchange chromatography[1] (Fig. 5).

Examination of the hydrolysis products indicates that two chemically related compounds with similar spectral data are formed rapidly and subsequently degraded to peak 3. The increase in absorbance of peak 3 equals exactly the loss of absorbance from peaks 1 and 2. This indicates there is no change in extinction coefficient, which leads us to suggest that alkali yields a symmetrical cleavage of the imidazolium ring, giving the 5-formyl and 6-formyl isomers, and that peak 3 presumably arises from the further hydrolysis of the formyl group to give N^6-ribosyl-5,6-diaminoisocytosine. The increased retention of peak 3 due to the liberated amino group is consistent with this hypothesis.

We thank Mr. L. L. TRIPLETT and Dr. A. H. HABER for performing the chlorophyll preservation assays. This research was sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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1 M. UZIEL, C. KOH AND W. E. COHN, *Anal. Biochem.*, 25 (1968) 77.
2 C. A. BURTIS, *J. Chromatogr.*, 51 (1970) 183.
3 R. L. HANCOCK, *J. Chromatogr. Sci.*, 7 (1969) 366.
4 M. JACOBSEN, J. F. OBRIEN AND C. HEDGCOTH, *Anal. Biochem.*, 25 (1968) 363.

5 B. H. MOST, J. C. WILLIAMS AND K. J. PARKER, *J. Chromatogr.*, 38 (1968) 136.
6 D. F. BABCOCK AND R. O. MORRIS, *Biochemistry*, 9 (1970) 3701.
7 F. SANGER, G. G. BROWNLEE AND B. G. BARRELL, *J. Mol. Biol.*, 13 (1965) 373.
8 S. NISHIMURA, F. HARADA, U. NARUSHIMA AND T. SENO, *Biochim. Biophys. Acta*, 142 (1967) 133.
9 A. D. KELMERS, H. O. WEEREN, J. F. WEISS, R. L. PEARSON, M. P. STULBERG AND G. D. NOVELLI, *Methods Enzymol.*, in press.
10 W. J. BURROUGHS, D. J. ARMSTRONG, F. SKOOG, S. M. HECHT, J. T. BOYLE, N. J. LEONARD AND J. OCCOLOWITZ, *Biochemistry*, 8 (1969) 3071.
11 B. I. S. SRIVASTAVA, *Int. Rev. Cytol.*, 22 (1967) 354.

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CHROM. 5342

Some aspects of the fractionation of DNA on an IR-120 Al^{3+} column

VI. The effect of pH and temperature variation on the chromatographic profiles of DNA

The shape, size and conformation of the DNA molecule as suggested by Watson and Crick's double helical model is largely dependent on a balance of opposing forces such as hydrogen bonding, Van der Waal's interionic forces, kinetic forces and varies with the environment. Thus, a change in the pH and/or temperature of DNA solution gives rise to changes in shape and rigidity *i.e.* three-dimensional structure of the molecule. DOTY AND RICE[1] have shown that lowering the pH and increasing the temperature results in structural alterations owing to breakage of hydrogen bonds. Structural transitions as a function of temperature and pH have also been observed by LUZZATI *et al.*[2] in the case of DNA and polyadenylic acid in solution. It was, therefore, thought worthwhile to investigate whether the variation in pH and temperature has any effect on the chromatographic behaviour, using an IR-120 Al^{3+} column.

DNA is quite unstable in the acidic pH range. Even at pH 6.0, it slowly starts apurinising. It is comparatively more stable in the alkaline range up to pH 10.6, because of the absence of a hydroxyl group in second position of the D-2-deoxyribose in DNA, which prevents cyclic phosphotriester formation prior to degradation, unlike that in the alkaline hydrolysis of RNA. Thus, the narrow pH range over which DNA is stable, limited the study of chromatographic behaviour at different pHs and was carried out only at pH 6.8, 8.6 and 10.0.

Experimental

IR-120 Al^{3+} column

10 g of dry regenerated Amberlite IR-120 (the Na^+ form of the cation exchanger) were equilibrated sufficiently with a 0.2 *M* aluminium chloride solution to give an IR-120 Al^{3+} column[3–8].

pH variation. Glycine–sodium hydroxide buffer at various pH, *viz.* pH 6.8, 8.6 and 10.0, was percolated through the column till the pHs of the influent and effluent were the same. The IR-120 Al^{3+} columns, thus equilibrated at the respective pHs, were then used for the fractionation studies.

Temperature variation. (1) Chromatographic studies were carried out at 4° by keeping the column in an Allwyn refrigerator. (2) The column was maintained at 27 ± 1° by the use of a jacketed column through which water from a thermostat was circulated by the peristaltic pump. The thermostat was adjusted to 27 ± 1° with the help of a mercury contact thermoregulator. A stirrer was used to avoid local variations in temperature. The relay used was from the Jumo Company. (3) The column was regulated at 42 ± 1° by means of a column condenser through which water at 42 ± 1° was circulated by a similar arrangement.

IR-120 Al^{3+} columns, equilibrated with glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) and maintained at 4, 27 and 42°, were then used for fractionation studies.

TABLE I

EFFECT OF pH VARIATION ON THE CHROMATOGRAPHIC PROFILES OF DNA ON AN IR-120 Al^{3+} COLUMN

Fraction eluted by	*Percent elution after adsorption of DNA carried out at pH*		
	6.8	*8.6*	*10.0*
Percent adsorption	90.0	100	90.0
Effluent	9.0	Nil	7.5
Buffer	Nil	Nil	2.5
0.5 *M* Saline	23.0	10.0	16.5
1.0 *M* Saline	4.0	15.0	1.0
2.0 *M* Saline	4.4	6.7	1.5
1.0% EDTA	47.0	33.0	60.0
2.0% EDTA	Nil	Nil	Nil
1.0 *M* Ammonium acetate	4.4	8.0	4.0
Distilled water	8.0	17.5	3.0
0.1 *M* Sodium hydroxide	Nil	10.0	3.0

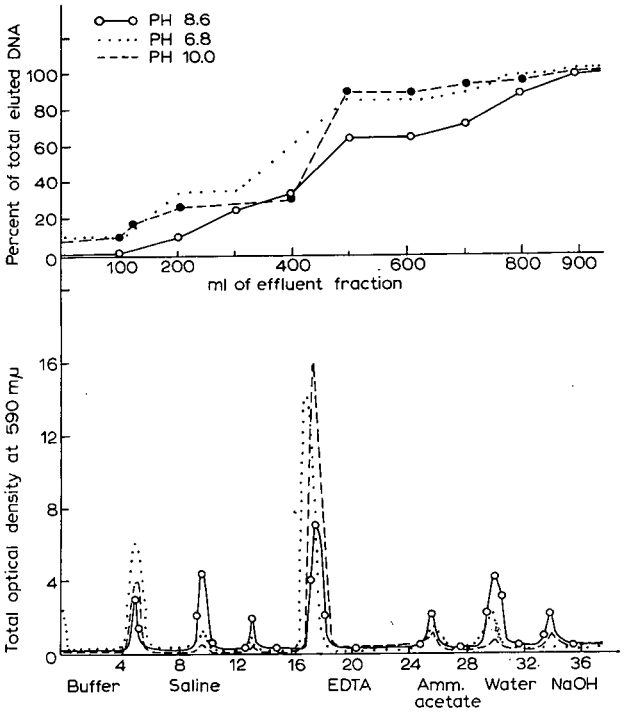

Fig. 1. Effect of pH variation on the chromatographic profiles of DNA on an IR-120 Al^{3+} column.

Deoxyribonucleic acid

The sodium salt of DNA, used in the present studies, was isolated from buffalo liver by the method of SEVAG *et al.*[9]. It was a white, fibrous and fairly pure preparation[4]. Its purity and nativity were examined by the usual methods[10]. It was devoid of any RNA contamination.

Procedure

A known amount of homogeneous DNA solution in glycine–sodium hydroxide buffers having various pHs, *viz.* pH 6.8, 8.6 and 10.0, was loaded on three separate IR-120 Al^{3+} columns, maintained at pH 6.8, 8.6 and 10.0, respectively. The adsorbed DNA was eluted with 100 ml of different eluting agents in the usual given sequence[4]. The flow rate during adsorption and elution was 10–15 ml per h. The fractions collected, each 25 ml, were assayed for their DNA content by BURTON's diphenylamine reaction[11].

TABLE II

EFFECT OF TEMPERATURE VARIATION ON THE CHROMATOGRAPHIC PROFILES OF DNA ON AN IR-120 Al^{3+} COLUMN

Fraction eluted by	*Percent elution after adsorption and elution carried out at*		
	4°	*27°*	*42°*
Percent adsorption	100	100	80.0
Effluent	Nil	Nil	2.0
Buffer	Nil	Nil	18.0
0.5 *M* Saline	18.0	10.0	20.0
1.0 *M* Saline	5.5	15.0	4.0
2.0 *M* Saline	15.0	7.0	1.6
1.0% EDTA	40.0	33.0	36.0
2.0% EDTA	Nil	Nil	Nil
1.0 *M* Ammonium acetate	7.0	8.0	6.5
Distilled water	12.0	17.0	6.0
0.1 *M* Sodium hydroxide	3.0	10.0	3.0

The effect of pH variation on adsorption and elution is given in Table I. Fig. 1 gives a graph of the percentage of total DNA eluted against different fractions obtained with 100 ml of different eluting agents. It also gives the elution profiles obtained, wherein the total optical density at 590 nm is plotted against the test-tube number or the fractions eluted.

A known amount of homogeneous DNA solution in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was chromatographed on three separate IR-120 Al^{3+} columns, previously equilibrated by the above buffer and maintained at three different temperatures, *viz.* 4, 27 and 42°.

The effect of temperature variation on adsorption and elution is given in Table II. Fig. 2 gives a graph of the percentage of total DNA eluted against different fractions obtained with 100 ml of different eluting agents. It also gives the elution profiles.

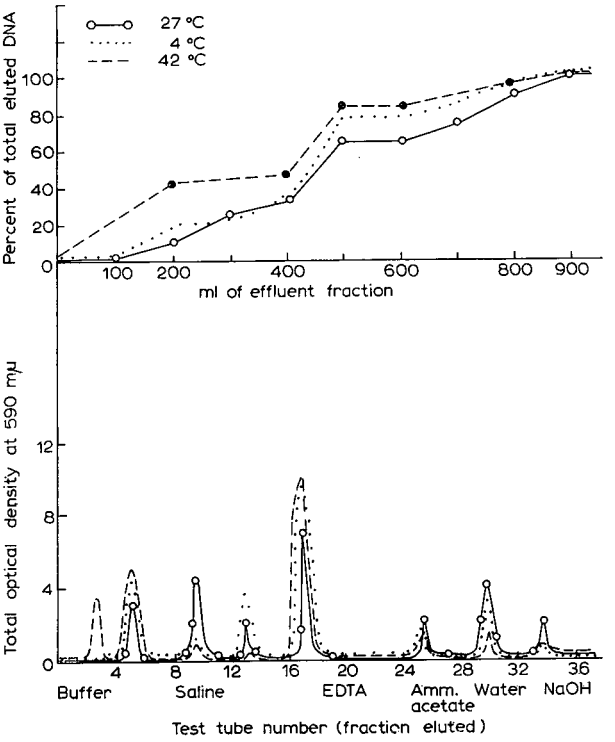

Fig. 2. Effect of temperature variation on the chromatographic profiles of DNA on an IR-120 Al^{3+} column.

Results and discussion

pH variation and profiles of DNA

Table I shows that there is 100% retention only at pH 8.6, and somewhat less retention at pH 6.8 and 10.0. The adsorbed DNA is nearly 100% eluted in each case and the nature of the elution profiles remains more or less the same. The percentage distribution of the DNA in the different fractions is, however, altered to some extent (Fig. 1).

A change in pH is known to bring about conformational changes in DNA[1,2]. MATHIESON AND MATTY[12] have noted the variation in intrinsic viscosity and streaming birefringence by changing the pH of the sodium salt of calf thymus DNA. In the present studies, however, no dramatic conformational changes in DNA can have taken place over the narrow pH range used, or else these would have been reflected in the chromatographic behaviour of DNA, as a definite conformation of DNA is necessary for its adsorption on an IR-120 Al^{3+} column[5,6,13].

Temperature variation and profiles of DNA

Table II shows that DNA is 100% retainable and 100% elutable at 4° and 27°. Only 80% of DNA is adsorbed at 42°, the remaining amount coming off in the effluent and buffer washing. The elution in this case is 95%. The nature of the elution profiles obtained at different temperatures is broadly the same. The percent distribution of

eluted DNA in the different fractions obtained shows minor modifications with variation in temperature (Fig. 2).

The present observations regarding the adsorption and elution of DNA at higher temperature, using an IR-120 Al^{3+} column, are comparable to those of MILLER[14] who showed that adsorption of native DNA at the water–mercury interphase is temperature dependent. The small variations observed in percentage distribution in profiles on the IR-120 Al^{3+} column may be due to easy dissociation of a complex between DNA and IR-120 Al^{3+}. MAY[15] has also shown that temperature influences the dissociation and formation of aggregates of thermally denatured DNA. Though elution at higher temperature may be quicker than at lower temperature, chromatography at a lower temperature is desirable as it reduces the risk of cleavage of labile bonds, and of bacterial or enzymatic degradation.

Structural transitions and retainability of DNA. The partial loss of retainability of DNA at 42° may be closely related to a partial change in the three-dimensional structure of the molecule. TIKCHONENKO *et al.*[16,17] have also shown that thermal denaturation of DNA proceeds through a state characterised by a number of properties intermediate between the rigid double-stranded and flexible single-stranded structures. This has been subsequently confirmed by BRAHMS AND MOMMAERTS[18] and is also supported by electron microscopic and viscometric studies[19] of the alterations introduced by the increased temperature in the secondary structure of DNA. The temperature of 42° is, however, much below the T_m of DNA studied, and therefore, the contribution of such a transition to the overall phenomenon is likely to be only marginal.

Effect of Mg^{2+} on the chromatographic behaviour of DNA at 42°. DNA effluent from an IR-120 Al^{3+} column at 42°, containing 20% nonretainable DNA, was also nonretainable at 27°. It was equilibrated with 0.001 *M* Mg^{2+} at 4° for 24 h and was again chromatographed on an IR-120 Al^{3+} column regulated at 42 and 27°. It was still found to be nonretainable at 42°, but retained at 27°.

It may be possible that at 42°, DNA is "demagnesised" resulting in an alteration of structure and subsequent loss in retainability. Mg^{2+} may be undertaking a "repair" of the "injury" caused by the higher temperature, attributing a finite three-dimensional structure acceptable to the IR-120 Al^{3+} column, hence causing retention at 27°. At 42°, however, DNA may once again undergo "demagnesisation" (dissociation) resulting in its nonretainability. It may be recalled here that the suggestion made by PEACOCKE[20] that the use of heat, acid and alkali be avoided, not only during isolation of DNA, but also in the subsequent physico-chemical studies, still holds to day.

Variation in the pH over which DNA is stable had no significant effect on the profiles. DNA was 100% retainable at 4 and 27°, but only 80% was retainable at 42°, the temperature considerably below the T_m. The elution profiles were quite comparable also at the different temperatures studied. Minor deviations observed in percent distribution in different fractions, as a result of pH and temperature variation, may be due to a partial alteration, if any, in the structure of DNA.

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1 P. DOTY AND S. A. RICE, *Biochim. Biophys. Acta*, 16 (1955) 446.
2 V. LUZZATI, A. MATHIS, F. MASSON AND J. WITZ, *J. Mol. Biol.*, 10 (1964) 28.
3 R. M. KOTHARI, *Chromatogr. Rev.*, 12 (1970) 127.
4 R. M. KOTHARI, *J. Chromatogr.*, 52 (1970) 119.
5 R. M. KOTHARI, *J. Chromatogr.*, 53 (1970) 580.
6 R. M. KOTHARI, *J. Chromatogr.*, 54 (1971) 239.
7 R. M. KOTHARI, *J. Chromatogr.*, 56 (1971) 151.
8 R. M. KOTHARI, *J. Chromatogr.*, 57 (1971) 83.
9 M. G. SEVAG, D. B. LACKMAN AND J. SMOLENS, *J. Biol. Chem.*, 124 (1938) 425.
10 R. M. KOTHARI, *Ph. D. Thesis*, Poona University, India, 1968.
11 K. BURTON, *Biochem. J.*, 62 (1956) 315.
12 A. R. MATHIESON AND S. MATTY, *J. Polym. Sci.*, 23 (1957) 747.
13 R. M. KOTHARI AND P. N. JOSHI, *Int. Symp. Genetics, Osmania University, Hyderabad (India). 1968*, Abstracts, 1968.
14 I. R. MILLER, *J. Mol. Biol.*, 3 (1961) 229.
15 P. MAY, *J. Mol. Biol.*, 9 (1964) 263.
16 T. I. TIKCHONENKO, G. A. PEREVERTAYLO AND E. N. DOBROV, *Biochim. Biophys. Acta*, 68 (1963) 500.
17 T. I. TIKCHONENKO, G. A. PEREVERTAYLO AND F. L. KISSELJOV, *Biochim. Biophys. Acta*, 76 (1963) 167.
18 J. BRAHMS AND W. F. H. M. MOMMAERTS, *J. Mol. Biol.*, 10 (1964) 73.
19 P. BARTL AND M. BOUBLIK, *Biochim. Biophys. Acta*, 103 (1965) 678.
20 A. R. PEACOCKE, *Biochim. Biophys. Acta*, 14 (1954) 157.

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CHROM. 5321

A quantitative protamine assay by diffusion in agarose gels containing DNA*

The determination of the concentration of protamine solutions for experimental, especially chromatographic, and for pharmaceutical purposes is complicated by several factors. Standard procedures, such as the Folin method or measurement of the extinction at 280 nm, cannot be employed (Fig. 1) because of the peculiar amino acid composition of these basic nuclear proteins. Kjeldahl determinations require special, time-consuming calibrations, as the N-content of the protamines is outside the normal protein range. Moreover they are non-immunogenic, so that immunological methods cannot be used.

A new assay for protamine, based on the diffusion of the sample in a layer of DNA-containing agarose and similar to the immunochemical technique used by

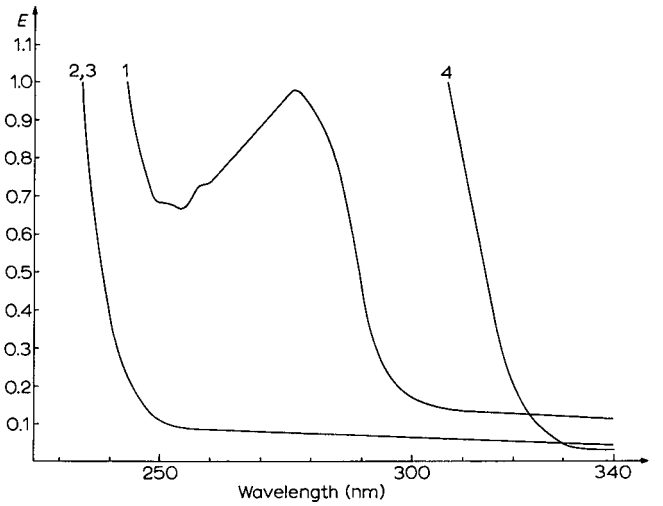

Fig. 1. UV-absorption curve of the protamine preparations investigated; the poly-lysine preparation and an albumin sample are included for comparison. 1 = albumin (Serva, human); 2 = salmine sulphate (Serva); 3 = poly-lysine HBr (Servá); 4 = "Protamine Roche" (pharmaceutical preparation of protamine HCl). Note the absence of the usual peak at 280 nm for 2 and 3 and the strong absorption for 4, possibly due to an additive for special purposes. Quantitative determination is not possible for 2, 3 and 4.

MANCINI[1] is described. Equal parts of a 2% solution of agarose (Behringwerke, Marburg, G.F.R.) and of a 0.2% solution of DNA (Serva, Heidelberg, G.F.R.; from salmon testes, high molecular), both in a pH 8.6 buffer, are thoroughly mixed at a temperature of 45°. 1.1 ml portions of this solution are evenly spread on carefully cleaned microscope slides (74 × 24 mm). After cooling, 2.6 mm diameter wells are punched out of the gel and the spare gel is removed. The slides are kept in a moist chamber at 4° until use.

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For the assay, 2 μl of the sample are placed in the wells by means of a microsyringe. After 3 h of diffusion in a moist chamber at 37° the diameters of the precipitation rings formed are determined by use of a calibrated loop. Diameters are measured twice at right angles and the mean value is employed for the assay. No dyeing procedure is needed as the precipitates formed are visible as thick, white haloes around the wells without further treatment. Comparison of the diameters with a standard curve (Fig. 2, No. 1) permits the concentration of the samples to be

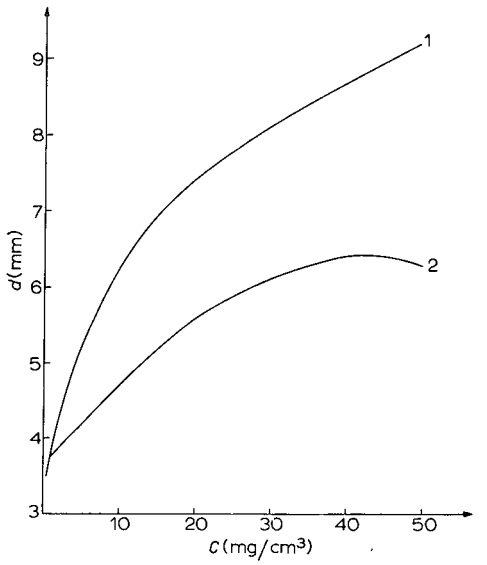

Fig. 2. Standard curves of the DNA-agarose diffusion method for "Protamine Roche" and salmine (No. 1) and for poly-lysine (No. 2).

read directly. The presence of blood plasma does not result in any visible precipitation rings; those formed by histone solutions are—under the experimental conditions described—not usable for concentration measurement. A standard curve for a poly-lysine preparation (Serva, mol. wt. = 100 000) is also included in the diagram (Fig. 2, No. 2). It is characterized by a peak at about 40 mg/cm³ and a decline at higher concentrations. Therefore the method described, in the case of poly-lysine, is only useful at lower concentrations.

It should be mentioned that agar gels are not suitable for the diffusion assay because all basic proteins are precipitated by the acid groups, especially SO_4^{2-}, present. They are precipitated in a diffuse way not comparable to the precipitates formed by DNA.

The technique described here provides an easy method of determination of protamine concentrations in a relatively short period of time from samples of only 2 μl.

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1 G. MANCINI, J.-P. VAERMAN, A. O. CARBONARA AND J. F. HEREMANS, in H. PEETERS (Editor), *Protides of the Biological Fluids*, Vol. II, Elsevier, Amsterdam, 1964, p. 370.

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CHROM. 5366

Identification par chromatographie sur couches minces des chlorhydrates des méthylamines et des éthylamines

La chromatographie des alkylamines sur colonne, sur couches minces ou sur papier, avec des solvants variés[1–16] sépare les termes en C_3 et au delà. Des difficultés, dûes surtout à l'adsorption des amines sur les supports, sont tournées en chromatographiant les dérivés acylés[14–24]: alors les premiers termes sont séparés. Mais nous cherchons une méthode de chromatographie directe, sans traitement préalable, afin de déceler des alkylamines en C_1 et C_2* que nous soupçonnons présentes dans les mélanges sels d'ammonium–méthanal[25]. Ces mélanges sont utilisés dans la formoltitration de RONCHESE où l'on devrait voir tout l'azote se transformer en hexaméthylènetétramine seule: en fait, une partie de l'azote se trouve combinée sous d'autres formes (imines, amines, etc.).

Partie expérimentale, chromatographie ascendante sur couches minces

Produits utilisés. Solvants et réactifs: Merck ou Prolabo. Chlorhydrates d'alkylamines Fluka et Carlo Erba. Le chlorhydrate de tri-*n*-butylamine est obtenu par neutralisation de la base en solution aqueuse.

Préparation des couches minces. Étaleur de Stahl à fente réglable (Desaga): 250 à 350 μm sur plaques de verre, longueur 200 mm.

Les différents supports employés sont: la terre siliceuse G pour CCM et le gel de silice G selon Stahl pour CCM (Merck), la poudre de cellulose pour CCM (Pleuger) et la poudre de cellulose MN 300 sans liant (Macherey et Nagel), la poudre de cellulose avec gypse pour CCM (Pleuger), les résines AG 50W-X8 pour CCM, 200–400 mesh, sphériques, avec liant et "Chelex 100", 200–400 mesh (Bio-Rad).

Composition des réactifs de révélation. R_1, ninhydrine 0.27 g, pyridine 5 ml, méthanol 95 ml; R_2, ninhydrine 0.27 g, pyridine 5 ml, DMSO 95 ml; R_3, naphtoquinone-1,2 sulfonate de sodium 0.60 g dissous dans 12 ml d'eau, ajouter éthanol 90° jusqu'à 200 ml et enfin 10 ml de pyridine; R_4, 1.5 g d'iode bisublimé dissous dans 250 ml d'éther de pétrole. R_1, R_2, R_3 sont employés en pulvérisation, R_4 en bain.

Lecture des chromatogrammes. Dans nos conditions opératoires, l'identification des taches demande que deux R_F voisins diffèrent au moins de 0.06 unité pour que ces taches soient absolument indépendantes.

Résultats et discussion

Efficacité de la révélation

Nous avons choisi nos quatre réactifs après essais de nombreux révélateurs proposés pour les amines. L'iode révèle les amines primaires, secondaires et tertiaires en les colorant identiquement. Par analogie nous avions essayé l'action du brome et du chlore sans aucun avantage.

La ninhydrine est beaucoup plus sensible que l'iode mais elle n'est pas plus spécifique des amines[26–28]. Elle ne doit pas révéler les amines tertiaires: seule BERTETTI[4] signale la possibilité d'une faible coloration avec celles-ci. Nous avons

* Abréviations utilisées dans ce mémoire: Me = méthylamine, DiMe = diméthylamine, TriMe = triméthylamine, E = éthylamine, DiE = diéthylamine, TriE = triéthylamine.

TABLEAU I

R_F DANS LE SOLVANT ALCOOL ISOAMYLIQUE–ACIDE CHLORHYDRIQUE SUR CELLULOSE SANS LIANT

Composition du solvant	*R*	*Me*	*DiMe*	*TriMe*	*E*	*DiE*	*TriE*	*Mélange*	*Temps de migration (h)*
100:40	R_1	0.34	0.42	[a]	0.48	0.75	[a]	0.34–0.45	13
	R_2	0.34	0.42	0.43	0.48	0.75	0.76	0.50–0.79	
	R_4	0.34	0.42	0.43	0.48	0.75	0.76		
150:30	R_1	0.28	0.38	[a]	0.45	0.73	[a]	0.28–0.40	9
								0.46–0.73	

[a] Amines tertiaires non révélées, $c \leqslant 20\%$.

chromatographié sur cellulose avec différents solvants (Tableaux I, II, V) les solutions aqueuses pures à 30% de chlorhydrate de TriMe, TriE, tributylamine et les mélanges de chlorhydrates de TriMe à 30% et de Me à 10% ou de DiMe à 20% et d'E à 10%. Les plaques sont sèchées à l'air normalement et pulvérisées avec les réactifs R_1 ou R_2, mises à l'étuve à 60° puis chauffées lentement jusqu'à 90–100°. Les trois amines tertiaires seules ou séparées de ces mélanges donnent des taches pâles distinctes jaunes, vertes ou bleues quelquefois incolores, mais parfaitement décelables cependant car les plaques de cellulose après traitement à la ninhydrine et chauffage présentent un fond très légèrement coloré en rose—traces d'alcool de la phase mobile[26]—et les taches tranchent nettement alors. Les R_F sont confirmés sur d'autres plaques révélées avec l'iode R_4.

TABLEAU II

R_F DANS LES SOLVANTS ALCOOLS VARIÉS–ACIDE CHLORHYDRIQUE SUR CELLULOSE SANS LIANT
Révélateur R_1.

Composition du solvant	*Me*	*DiMe*	*TriMe*	*E*	*DiE*	*Mélange*	*Temps de migration (h)*
Alcool heptylique normal–HCl (100:13)	0.12	0.15	—	0.22	0.46	0.10–0.15 0.21–0.47	9
Alcool heptylique normal–HCl (100:45) (saturation)	0.29	0.38	0.34[a]	0.43	0.64	0.27–0.37 0.43–0.64	19½
Alcool isoamylique–alcool heptylique–HCl (10:50:15)	0.22	0.29	—	0.37	0.63	0.22–0.30 0.37–0.63	9
Alcool isoamylique–alcool octylique secondaire–HCl (1:2:1)	0.24	0.32	—	0.39	0.64	0.24–0.32 0.37–0.63	10½

[a] Révélateur R_2.

Après chromatographie bidimensionnelle des solutions aqueuses à 30% des amines tertiaires précédentes, la ninhydrine ne révèle plus que la tri-*n*-butylamine: la dilution des taches après deux migrations abaisse trop la concentration pour que les amines tertiaires légères soient encore révélées. Donc les amines tertiaires sont détectées par la ninhydrine quand leur concentration atteint un seuil suffisant. Le Tableau III résume le comportement des six amines vis à vis des réactifs de révélation utilisés.

TABLEAU III

Durée de vie des colorations des taches[b]: R_1 et R_3, plusieurs jours; R_2, plusieurs jours, maximum d'intensité quelquefois au bout d'un jour; R_4, quelques minutes. Couleur du fond (phase fixe)[a,b]: R_1 et R_2 incolore ou légèrement rosé. Peut devenir violet quand la température de révélation dépasse 90°; les taches sont alors visibles car elles se décolorent; R_3, jaune; R_4, jaune pâlissant très rapidement.

Alkylamines C_1 *et* C_2	*Couleur des taches*[a,b]				*Intensité relative des taches*[b,c]		
	R_1	R_2	R_3	R_4	R_1, R_2	R_3	R_4
Me	jaune, gris, bleu à violet	vert pré, violet, gris	rose soutenu	jaune à orange	+++	+±	+
DiMe					+±	+	+
TriMe	incolore, jaune pâle, vert pâle, violet pâle		o	jaune à orange	±	o	+++
E	jaune, bleu foncé à violet	vert pré, violet foncé	violet pâle	jaune à orange	++++	+++	++
DiE	jaune, bleu pâle	vert pré, bleu-violet	rose à peine visible	jaune à orange	+	±	+
TriE	incolore, jaune pâle, vert léger		o	orange	±	o	+++

[a] En lumière blanche.
[b] Appréciation subjective à l'oeil.
[c] o, aucune coloration; ±; +; +±; ++; +++; ++++; coloration d'intensité croissante.

Séparation en milieu acide

Sur cellulose sans liant. Un éluant ternaire, acide–alcool–eau sépare quatre amines sur six. Avec l'acide acétique les taches sont brouillées par des altérations de couleur plus faible en deça ou au delà de la tache. Le mélange *n*-butanol–acide chlorhydrique–eau a l'avantage sur le précédent de donner de belles taches sans trainée. Avec l'alcool isoamylique au lieu de butanol la proportion optimale d'acide chlorhydrique est de 28.6% en volume d'acide pur ($d = 1.19$). La séparation est meilleure (Tableau I).

On observe que ce sont les alcools les plus "lourds" (heptylique, octylique etc.) qui assurent la meilleure séparation, mais ils augmentent le temps de chromatographie et de séchage des plaques (Tableau II).

Avec les meilleurs solvants (voir Tableaux I et II), la chromatographie du mélange des six chlorhydrates d'amines—0.20 μl contenant 7 μg de chaque amine primaire, 15 μg de chaque secondaire et 20 μg de chaque tertiaire—permet la séparation et la caractérisation de la Me, DiMe, E, DiE, en révélant les chromatogrammes avec les réactifs R_1 ou R_2. TriMe et TriE en quantité insuffisante ne sont pas révélées. Mais leurs R_F déterminés avec l'iode sont très voisins de ceux des amines secondaires.

Autres supports. Sur terre siliceuse G, les amines sont moins retenues que sur cellulose; la séparation est du même genre mais beaucoup moins efficace et moins

TABLEAU IV

R_F SUR GEL DE SILICE

Révélateur R_2. Couleurs des taches: Me = violet foncé; DiMe = violet foncé; TriMe = jaune, vert, bleu; E = violet foncé; DiE = bleu foncé à violet; TriE = jaune.

Solvant	*Me*	*DiMe*	*TriMe*	*E*	*DiE*	*TriE*	*Mélanges*	*Temps (h)*
alcool isoamylique–HCl (100:40)	0.32	0.26	0.22	0.37	0.35	0.31	0.22–0.30 0.37	12
alcool heptylique normal–HCl (100:40)	0.22	0.16	0.12	0.25	0.23	0.16	0.12–0.17 0.23	20
alcool heptylique normal–HCl (100:45)	0.22	0.14	0.10	0.23	0.20	0.15	0.11–0.16 0.23	17

belle avec des trainées et deux fronts de solvant. Par contre le gel de silice G, avec les mêmes solvants permet de séparer la TriMe dans le mélange des six amines comme le montre le Tableau IV. Il reste le problème de la TriE qui est masquée quelque soit le support et le solvant acide employés.

Remarquons enfin que les amines sont retenues par la phase fixe dans l'ordre suivant: Terre siliceuse G < cellulose < gel de silice.

Chromatographie d'échange. La méthode de séparation des ions monovalents[29–37] sur résine AG 50W-X8, éluée par l'acide nitrique 1/6, n'est pas efficace pour les chlorhydrates de nos amines.

Sur support de poudre de cellulose imprégnée d'acide silico-12 tungstique en solution aqueuse à 5%, séchée à l'air, puis éluée par l'alcool isoamylique–acide chlorhydrique (100:40), les résultats sont assez identiques à ceux de la chromatographie sur cellulose pure.

Sur cellulose mélangée avec de la résine "Chelex 100", préalablement saturée en cuivre II, (cellulose résine: 1/6 en poids) les amines après migration donnent directement des taches jaunes sur fond vert clair mais la séparation est mauvaise même avec le solvant précédent.

La cellulose imprégnée de cuivre II ou de nickel—solution aqueuse des chlorures[38–40]—fournit aussi dès sèchage de la plaque chromatographiée des taches

TABLEAU V

R_F DANS LE SOLVANT *n*-BUTANOL–PYRIDINE–EAU SUR CELLULOSE SANS LIANT

Révélateur R_1. Couleurs des taches: Me = gris foncé (c), violet (e) jaune + anneau violet (a); DiMe = jaune pâle (c), jaune clair (a); TriMe = bleu pâle (c), violet pâle (a); E = bleu foncé (c); violet (e), jaune + anneau violet (a); DiE = bleu foncé (c), jaune clair (a).

Composition du solvant	*Me*	*DiMe*	*TriMe*	*E*	*DiE*	*Mélange*	*Temps de migration*	*Observations*
(a) 30:90:30	0.48	0.50	0.46	0.55	0.66	—	3 h	taches rondes sans traînés
(b) 30:108:30	0.42	0.46	0.42	0.49	0.66	0.40–0.45 0.52–0.66	1 h 25 min	taches ovales sans traînée
(c) 30:120:30	0.42	0.53	0.48	0.59	0.73	0.46–0.53 0.60–0.72	1 h 30 min	taches ovales sans traînée
(d) 30:138:30	0.38	0.42	0.38	0.48	0.63	0.38–0.43 0.49–0.62	1 h 25 min	taches rondes sans traînée
(e) 20:100:30	0.46	0.49	0.45	0.53	0.66	0.43–0.49 0.55–0.66	3 h 50 min	taches ovales sans traînée

colorées (jaunes sur fond vert dans le cas du cuivre, bleues sur fond blanc dans le cas du nickel), même pour les amines tertiaires. Les amines forment donc des complexes relativement stables avec ces ions métalliques même en milieu fortement acide. La résolution du mélange est identique à celle sur cellulose pure (Tableau I). Dans nos conditions opératoires, le cuivre et le nickel ne semblent jouer qu'un rôle de révélateur.

Séparation en milieu alcalin

Nous avons utilisé le mélange *n*-butanol–pyridine–eau (30:90:30) employé pour la séparation des amino-acides[41]. Pour comparer ses qualités avec celles des solvants acides nous faisons varier sa composition. Les variations des teneurs en alcool ou en eau sont défavorables: il faut garder le rapport alcool–eau (1:1). La teneur optimale en pyridine apparaît dans le mélange (30:120:30) sans cependant séparer parfaitement DiMe et TriMe,—R_F 0.53 et 0.48—(Tableau V). L'ordre

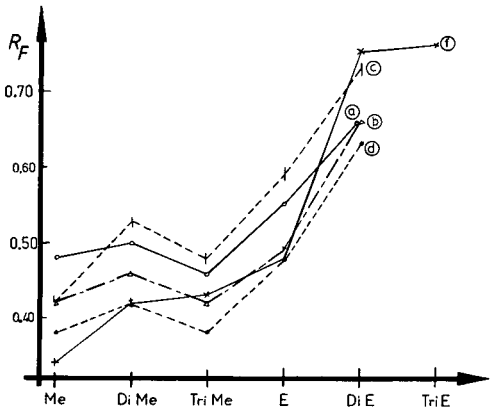

Fig. 1. R_F comparés des amines sur cellulose en milieu acide ou alcalin. Solvants a, b, c et d, voir Tableau V; f, alcool isoamylique–acide chlorhydrique (100:40).

croissant des R_F s'établit alors: Me < TriMe < DiMe < E < DiE. La TriE donne le même R_F que la DiE. Ce milieu alcalin est donc égal et même légèrement supérieur aux meilleures phases acides en considérant les valeurs relatives des R_F (Figure 1).

Conclusions

La séparation de Me, DiMe, E, DiE est obtenue sur cellulose sans liant en milieu acide, par exemple alcool isoamylique–acide chlorhydrique (100:40) ou en milieu alcalin *n*-butanol–pyridine–eau (30:120:30).

En présence d'amines tertiaires en faible quantité la séparation s'effectuera de même, sans gêne pour la révélation à la ninhydrine.

Avec la TriMe en forte quantité on identifiera les cinq amines sur cellulose en milieu alcalin puis la TriMe sera confirmée par chromatographie sur gel de silice avec un solvant acide et révélation à la ninhydrine avec chauffage très progressif.

La TriE donne le même R_F que la DiE sur cellulose ou la DiMe sur gel de silice, on ne peut donc pas l'identifier. Dans le cas où sa concentration lui permettrait d'être révélée par la ninhydrine et de gêner l'identification de la DiE ou de la DiMe

une chromatographie bidimensionnelle résoudra cette difficulté puisqu'alors la TriE diluée ne sera plus révélée.

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1 J. M. BREMNER ET R. H. KENTEN, *Biochem. J.*, 49 (1951) 651.
2 R. SCHWYZER, *Acta Chem. Scand.*, 6 (1952) 219.
3 M. STEINER ET E. STEIN VON KAMIENSKI, *Naturwissenschaften*, 40 (1953) 483.
4 J. BERTETTI, *Ann. Chim. (Rome)*, 43 (1953) 351 et 361.
5 W. DIHLMANN, *Biochem. Z.*, 325 (1954) 295.
6 K. TEICHERT, E. MUTSCHLER ET H. ROCHELMEYER, *Deut. Apoth. Ztg.*, 100 (1960) 283.
7 E. MERCK AG, *Chromatographie*, 85–86.
8 E. LEDERER, *Chromatographie en Chimie Organique et Biologique*, Vol. 1, Masson, Paris, 1959, 78–81, 497–498.
9 L. MEITES, *Handbook of Analytical Chemistry*, 1e éd., Mc Graw-Hill, New York, Toronto, London, 1963, pp. 10–73.
10 J. M. BOBITT, *Thin-Layer Chromatography*, Reinhold, New York, London, 1963, p. 133.
11 H. GRASSHOF, *J. Chromatogr.*, 20 (1965) 163.
12 D. MIKEŠ, *Laboratory Handbook of Chromatographic Methods*, D. van Nostrand, London, 1966, pp. 127–128.
13 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
14 K. RANDERATH, *Thin-Layer Chromatography*, Verlag Chemie Academic Press, New York, London, 1963, pp. 90–91.
15 J. G. KIRCHNER, *Thin-Layer Chromatography*, Interscience, J. WILEY & Sons, New York, London, Sydney, 1967, pp. 293–295.
16 E. STAHL, *Thin-Layer Chromatography*, 2e éd., Springer, Berlin, Heidelberg, New York, 1969, pp. 494–496.
17 S. PATAI, *The Chemistry of the Amino Group*, Interscience, J. Wiley & Sons, London, New York, Sydney, 1968, p. 98.
18 G. NEURATH ET E. DOERK, *Chem. Ber.*, 97 (1964) 172.
19 N. SEILER ET M. WIECHMANN, *Experientia*, 21 (1965) 203.
20 T. MOMOSE ET M. NAKAMURA, *Chem. Pharm. Bull. Jap.*, 10 (1962) 553.
21 A. JART ET A. J. BIGLER, *J. Chromatogr.*, 29 (1967) 255.
22 H. VAN DUIN, *Biochim. Biophys. Acta.*, 12 (1953) 490.
23 I. M. LOCKHART, *Nature*, 177 (1956) 393.
24 D. B. PARIHAR, S. P. SHARMA ET K. K. VERMA, *J. Chromatogr.*, 26 (1967) 292.
25 PH. BAUDOT, *Chim. Anal.*, 51 (1969) 425.
26 J. L. GAUMET, *Thèse*, Faculté des Sciences, Bordeaux, 1969.
27 D. GROSS, *J. Chromatogr.*, 10 (1963) 233.
28 E. D. SCHILLING, P. I. BURCHILL ET R. A. CLAYTON, *Anal. Biochem.*, 5 (1963) 1.
29 E. HEFTMANN, *Chromatography*, Reinhold, New York, 1961, pp. 285–288, 321–322.
30 L. MEITES, *Handbook of Analytical Chemistry*, 1e éd., Mc Graw-Hill, New York, Toronto, London, 1963, pp. 10–159 et 164.
31 J. C. GIDDINGS ET R. A. KELLER, *Adv. Chromatogr.*, 20 (1965) 1 et 34.
32 D. MIKEŠ, *Laboratory Handbook of Chromatographic Methods*, D. van Nostrand, London, 1966, p. 284.
33 J. A. BERGER, G. MEYNIEL ET J. PETIT, *J. Chromatogr.*, 29 (1967) 195.
34 J. G. KIRCHNER, *Thin-Layer Chromatography, Interscience*, J. Wiley & Sons, New York, London, Sydney, 1967, pp. 721–722.
35 F. W. E. STRELOW, J. H. J. COETZEE ET C. R. VAN ZYL, *Anal. Chem.*, 40 (1968) 196.
36 L. L. WILSON ET D. W. WILSON, *Comprehensive Analytical Chemistry*, Vol. 11B, Elsevier, Amsterdam, 1968, pp. 254–257.
37 G. L. STAROBINETS, A. B. CHIZHEVSKAYA, R. V. MARTSINKEVICH ET L. M. DVSYANKO, *Teor. Innogo Obmena Khromatogr., Tr. Vses. Nauch.-Tekh. Konf.*, (1965) 78. *C.A.*, 71 (1969) 53990v.
38 F. HELFFERICH, *Nature*, 189 (1961) 1001.
39 K. SHIMOMURA, L. DICKSON ET H. F. WALTON, *Anal. Chim. Acta*, 37 (1967) 102.
40 R. A. A. MUZZARELLI, A. FERRERO MARTELLI ET O. TUBERTINI, *Analyst*, 94 (1969) 616.
41 R. MUNIER, C. THOMMEGAY ET G. SARRAZIN, *Bull. Soc. Chim.*, 10 (1967) 3971.

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CHROM. 5364

Gaseous formaldehyde: A sensitive chromatographic detection reagent for tryptophanyl-dipeptides

A few years ago, formaldehyde gas was introduced as a sensitive and specific chromatographic detection reagent for catecholamines and indoleamines[1–8]. After condensation with formaldehyde, these amines, or rather their condensation products, were found to emit strong fluorescence in UV light. The condensation procedure used for the chromatographic analysis was essentially the same as that described earlier for the fluorescence microscopic localization of these biogenic arylethylamines[9–12]. The chemical reactions that result in fluorophore formation have been investigated in some detail[10–13]. Briefly, the concept is as follows: in an initial reaction step, the catecholamines and indoleamines are condensed with formaldehyde, forming tetrahydroisoquinolines and tetrahydro-β-carbolines, respectively. In a second step, these derivatives are oxidized (by dehydrogenation) to highly fluorescent dihydroisoquinolines and dihydro-β-carbolines. The precise chemical identities of the resulting fluorophores are unknown. Recent evidence suggests that a number of different formaldehyde-induced fluorophores may result from each individual amine[14].

Apart from catecholamines and indoleamines, certain amino acids with a catechol or indole group also emit strong fluorescence upon formaldehyde treatment. Tryptophan is one of the indoleamino acids which readily form a fluorophore with formaldehyde. Also tryptophanyl-peptides (with a free tryptophan amino group) might be expected to react with formaldehyde, giving rise to fluorescent conjugates. This assumption was confirmed in the present investigation. Certain tryptophan-containing dipeptides were found to give intense fluorescence on silica gel thin layers after formaldehyde treatment, permitting the chromatographic detection of small amounts of these compounds.

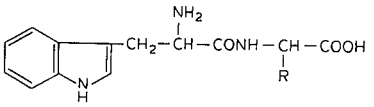
Experimental

Silica gel thin layers were prepared by coating glass cover slips (24 × 32 mm, for routine histology) with approximately 100-μm Kieselgel H (Merck, Darmstadt). The layer was applied as a slurry consisting of 20 g silica gel suspended in 50 ml glass-distilled water. The plates were dried at room temperature before use. Aqueous solutions of tryptamine hydrochloride, tryptophan and tryptophan-containing peptides (see Table I) in various concentrations were applied to the thin layers in volumes of 0.5 μl. The silica gel thin layers were exposed to formaldehyde gas generated from paraformaldehyde (equilibrated in an atmosphere of about 50% relative humidity) at 100° for 30–60 min. The thin layers were examined in UV light (Sterisol UV-lamp, Original Hanau, equipped with a UG 1 filter) and the fluorescence was analyzed in a modified Leitz microspectrograph[15]. The fluorescence intensities of the various spots were visually evaluated in the UV light; the minimum detectable amount was used as a measure of the fluorescence intensity. For the microspectrofluorometric analysis, the optical system for the exciting light consisted of quartz components, and the glass cover slips were placed upside down with the thin layer facing the condenser. The thin layer outside the fluorescent spot was used to obtain blank spectra. All spectra were corrected for instrumental errors according to procedures previously

described[15]. All values given for excitation and emission maxima are the mean values of at least four separate determinations.

Results and discussion

All the tryptophanyl-peptides tested gave strong greenish-yellow fluorescence upon formaldehyde treatment. As a chromatographic detection reagent formaldehyde gas had a similar sensitivity for tryptophanyl-dipeptides as for tryptamine and tryptophan. The minimum detectable amount varied from 0.3 to 0.1 μg for the different tryptophanyl-dipeptides (Table I). None of the tryptophan-containing peptides having the tryptophan amino group engaged in peptide linkage, gave visible fluorescence with formaldehyde. Thus, the dipeptides that gave strong formaldehyde-induced fluorescence had the following general formula:



The excitation and emission maxima of the various tryptophanyl-dipeptides were remarkably similar: excitation at 375–380 mμ, emission at approximately 500 mμ (Table I). The second amino acid in the dipeptide appeared to have only a minor influence on the spectral properties of the fluorophore. It should be noted that the spectral properties of the fluorophores of the tryptophanyl-dipeptides showed a fairly close resemblance to those of the tryptamine fluorophore while differing from those of the tryptophan fluorophore (Fig. 1).

TABLE I

FORMALDEHYDE-INDUCED FLUORESCENCE OF TRYPTAMINE, TRYPTOPHAN AND SOME TRYPTOPHAN-CONTAINING DIPEPTIDES ON SILICA GEL

All peptides were purchased from Miles Laboratories, Ill., U.S.A.

Compounds	*Minimum detectable amount (μg)*	*Excitation/emission max. (mμ)*
Tryptamine	0.01	370/500
L-Tryptophan	0.03	375/450
L-Tryptophanyl-L-alanine	0.1	375/500
L-Tryptophanyl-L-glycine	0.1	375/500
L-Tryptophanyl-L-tyrosine	0.3	380/500
L-Tryptophanyl-L-tryptophan	0.3	380/505
L-Glycyl-L-tryptophan	Non-detectable	
L-Phenylalanyl-L-tryptophan	Non-detectable	
L-Propyl-L-tryptophan	Non-detectable	

Conceivably, all peptides with N-terminal tryptophan residues give fluorophores upon formaldehyde condensation. The results strongly suggest that also peptides with N-terminal 3,4-dihydroxyphenylalanine or 5-hydroxytryptophan residues can be expected to emit formaldehyde-induced fluorescence.

The method has been adapted also for the fluorescence histochemical demonstration of peptides and proteins with N-terminal tryptophan residues[16].

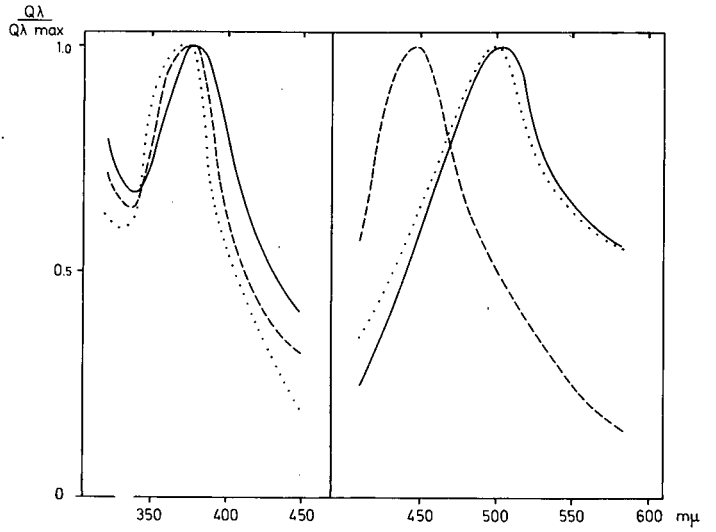


Fig. 1. Excitation (left) and emission (right) spectra of the formaldehyde-induced fluorophores of tryptamine (. . .), L-tryptophan (- - -) and L-tryptophanyl-L-glycine (——). Spectra were recorded from spots containing 0.3 $\mu g/cm^2$ of the indole and are expressed as relative quanta *versus* wavelength.

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1 C. E. BELL AND A. R. SOMERVILLE, *Biochem. J.*, 98 (1966) 1c.
2 R. HÅKANSON, M. N. LOMBARD DES GOUTTES AND CH. OWMAN, *Life Sci.*, 6 (1967) 2577.
3 D. AURES, R. FLEMING AND R. HÅKANSON, *J. Chromatogr.*, 33 (1968) 480.
4 E. J. COWLES, G. M. CHRISTENSEN AND A. C. HILDING, *J. Chromatogr.*, 35 (1968) 389.
5 D. AURES, A. BJÖRKLUND AND R. HÅKANSON, *Z. Anal. Chem.*, 243 (1968) 389.
6 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *J. Chromatogr.*, 40 (1969) 186.
7 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *J. Chromatogr.*, 47 (1970) 530.
8 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *Anal. Biochem.*, 35 (1970) 264.
9 B. FALCK, *Acta Physiol. Scand.*, 56 (1962) Suppl. 197.
10 B. FALCK, N. Å. HILLARP, G. THIEME AND A. TORP, *J. Histochem. Cytochem.*, 10 (1962) 348.
11 H. CORRODI AND N. Å. HILLARP, *Helv. Chim. Acta*, 46 (1963) 2425.
12 H. CORRODI AND N. Å. HILLARP, *Helv. Chim. Acta*, 47 (1964) 911.
13 G. JONSSON, *The Formaldehyde Fluorescence Method for the Histochemical Demonstration of Biogenic Monoamines*, Haeggströms Tryckeri AB, Stockholm, 1967.
14 S. AXELSSON, A. BJÖRKLUND AND R. HÅKANSON, to be published.
15 A. BJÖRKLUND, B. EHINGER AND B. FALCK, *J. Histochem. Cytochem.*, 16 (1968) 262.
16 R. HÅKANSON AND F. SUNDLER, *J. Histochem. Cytochem.*, (1971) in press.

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CHROM. 5373

Calciumcarbonat als Sorptionsmittel für die Dünnschichtchromatographie von Morphin- und Morphinanderivaten

Üblicherweise werden zur Dünnschichtchromatographie (DC) vornehmlich standardisiertes Aluminiumoxid oder Kieselgel verwendet. Diese Sorptionsmittel zeichnen sich vor allem durch gute Reproduzierbarkeit der Ergebnisse aus. Für die quantitative Bestimmung der getrennten Substanzen ist aber von Nachteil, dass in den meisten Fällen kein vollständiges Eluieren des ausgeschabten Fleckes möglich ist, so dass sich zwar gut reproduzierbare, aber immer zu niedrige Werte ergeben. Aus diesem Grund wäre die Verwendung löslicher Sorptionsmittel wünschenswert. In der Literatur finden sich bislang relativ wenige Arbeiten, die sich mit solchen Sorptionsmitteln befassen. RAGAZZI *et al.*[1] trennten erfolgreich die Alkaloide Chinin, Hydrastin und Reserpin mit DC auf hydratisiertem Magnesiumoxid, KEEFER[2] auf dem gleichen Sorptionsmittel polynucleare Aromaten. KIRCHNER *et al.*[3] untersuchten verschiedene basische säurelösliche Sorptionsmittel im Hinblick auf die Trennung von Bestandteilen ätherischer Öle. Systematische Untersuchungen an MgO, $Mg(OH)_2$ und $MgCO_3$ wurden von KARTNIG *et al.*[4] durchgeführt. Auf Calciumcarbonat wurden bislang unseres Wissens lediglich Kohlenhydrate[5] sowie Chloroplastenpigmente[6] getrennt.

Experimentelles

In unseren Untersuchungen stellten wir fest, dass neben Magnesiumoxid sich vor allem Calciumcarbonat für die DC von Alkaloiden eignet. Magnesiumcarbonat dagegen ergibt durchweg schlechtere Trennungen und bedingt ziemlich grosse Laufzeiten.

Im Handel befindet sich zur Zeit noch kein standardisiertes Calciumcarbonat, deshalb wurde handelsübliches $CaCO_3$ (Merck Art. Nr. 2066) verwendet. Es empfiehlt sich, um kleine Klümpchen zu entfernen, das Handelspräparat durch ein feines Sieb zu bürsten. Da eine Reihe organischer Lösungsmittel oftmals ein Abblättern der Sorptionsschicht bewirkt, wurde versucht, durch einen Zusatz von Gips haltbarere Schichten zu erzielen. Hier zeigte es sich, dass Zusätze bis etwa 5% praktisch keinen Einfluss auf die Trennleistung haben, während sich die Haltbarkeit verbessert. Als Aufschlämmflüssigkeit wurden Wasser, Methanol und auch andere organische Lösungsmittel versucht, wobei sich Wasser oder noch besser eine 2.5%ige Lösung von Calciumchlorid in Wasser als am geeignetsten erwiesen. Bei Gipszusatz ist es unbedingt zu empfehlen, zur Aufschlämmung Methanol–Wasser zu verwenden. Zur Herstellung möglichst gleichmässiger Schichten wurde die Calciumcarbonatsuspension etwa eine halbe Stunde lang mit einem Ultraturrax-Gerät homogenisiert.

Setzt man dem Sorptionsmittel Fluoreszensindikatoren zu, lassen sich die getrockneten Platten im UV-Licht auswerten. Ansonsten kann man zur Sichtbarmachung der Alkaloide auch Dragendorffs Reagens verwenden. Hierbei empfiehlt es sich, die Platten kurz zu besprühen und dann 20 min bei 60° zu entwickeln und nochmals kurz nachzusprühen.

Methodik. Herstellung der Platten: (A) 160 g Calciumcarbonat, 180 ml 2.5%ige Calciumchloridlösung und 3.5 g Fluoreszensleuchtstoff grün (Fa. Woelm) (für *ca.*

15 Platten 20 × 20 cm); (B) 155 g Calciumcarbonat; 5 g Gips; 3.5 g Fluoreszensleuchtstoff grün (Fa. Woelm); 40 ml Methanol; 125 ml 2.5%ige Calciumchloridlösung (für *ca.* 15 Platten 20 × 20 cm). Die Suspension wird *ca.* 20–30 min mit einem Ultraturrax homogenisiert. Die Platten werden über Nacht getrocknet.

Fliessmittel: Chloroform–Acetessigester (1:1). Detektion: UV-Lampe; Dragendorffs Reagens.

Ergebnisse

Die beste Trennung wurde mit dem Fliessmittel Chloroform–Acetessigester (1:1) erzielt. Daneben können auch Kombinationen von Tetrachlorkohlenstoff–Acetessigester verwendet werden. Zur Trennung von Pethidin und Methadon eignet sich das Fliessmittel Äthanol–Benzol (1:4). Die hR_F-Werte betragen 24 bzw. 80. Narcotin und Papaverin konnten in keinem Fliessmittelsystem eindeutig getrennt werden. Eine Erhöhung des unpolaren Anteils des Fliessmittels oder ein Zusatz von Benzol bewirkte eine Schwanzbildung der Flecken. Die Steighöhe beträgt jeweils 15 cm. Die in der Tabelle I angegebenen hR_F-Werte können nur als Anhaltspunkt

TABELLE I

hR_F-WERTE VON MORPHIN- UND MORPHINANDERIVATEN AUF $CaCO_3$–DC

Substanz	*hR_F im Fliessmittel Acetessigester–Chloroform (1:1)*
Morphin	13
Hydromorphon	22
Oxycodon	33
Codein	56
Dihydrocodein	62
Hydrocodon	71
Thebacon	82
Pethidin	88
Methadon	88
Narcotin	93
Papaverin	93
Eupaverin	97

dienen, da die einzelnen $CaCO_3$-Chargen unterschiedlich ausfallen und somit auch die Trennleistung nicht unbedingt reproduzierbar ist.

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1 E. RAGAZZI, G. VERONESE UND C. GIACOBAZZI, in G. B. MARINI-BETTÒLO (Editor), *Thin-Layer Chromatography*, Elsevier, Amsterdam, 1964, S. 149 *ff.*
2 L. K. KEEFER, *J. Chromatogr.*, 31 (1967) 390.
3 J. G. KIRCHNER, J. M. MILLER UND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
4 T. KARTNIG, A. HIERMANN UND G. MIKULA, *Pharm. Zentralh.*, 108 (1969) 177.
5 G. STEHLIK, *Symposium V, Chromatographie, Elektrophorèse, Bruxelles, 1969*, pp. 524–528.
6 H. H. STRAIN, J. SHERMA UND M. GRANDOLFO, *Anal. Biochem.*, 24 (1968) 54.

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CHROM. 5402

Thin-layer chromatography of caffeine and related methylxanthines

In connection with a research project on the metabolism of caffeine by microorganisms[1], we have tested a number of methods for the separation of methylxanthines by thin-layer chromatography[2–14]. Finding them wanting with respect to either convenience, speed, resolution, or sensitivity, we have developed a new procedure, which we are successfully using to isolate and identify radioactive metabolites in fungi and which is undoubtedly also applicable to other biochemical systems and to quantitative analysis of foods and beverages as well as pharmaceuticals during and after processing.

Reference compounds

Xanthine (X), hypoxanthine (H), 7-methylxanthine (7), 1-methylxanthine (1) and theobromine (3,7-dimethylxanthine, 37) were from Pfalz and Bauer, Flushing, New York*. Paraxanthine (1,7-dimethylxanthine, 17) was a gift of Mrs. R. N. WARREN, London Hospital Medical College, London, Great Britain, which is gratefully acknowledged. Theophylline (1,3-dimethylxanthine, 13) (aminophylline, theophylline$_2$ ethylenediamine), was from Sigma Chemical Corp., St. Louis, Mo. Caffeine (1,3,7-trimethylxanthine, C) was from J. T. Baker Chemical Co., Phillipsburg, N.J.

Solutions of 1 mg of each of the above compounds per ml were prepared with 0.25 *N* NaOH, except in the case of caffeine, which gradually decomposes in NaOH solution, and aminophylline, which is poorly soluble. Caffeine was dissolved in chloroform (1 mg/ml) and aminophylline in 95% aqueous ethanol (1.17 mg/ml).

Solvents

All solvents were 99+ mole % pure (spectroquality, Matheson Coleman and Bell, Los Angeles, Calif.). Three solvent systems are recommended: chloroform–methanol (4:1); ethyl acetate–methanol–ammonium hydroxide (8:2:1) (concentrated (28%) ammonia, Allied Chemicals Division, Morristown, N.J.); *n*-butanol, saturated with a 2.8% aqueous solution of ammonium hydroxide.

Procedure

50 ml of solvent were placed in a flat-bottom chromatography jar 23 × 23 × 8 cm. Prescored glass plates (Uniplates, Analtech, Inc., Newark, Del.), 20 × 20 cm, precoated with layers of Silica Gel GF 0.25 mm thick, were split in half. A finish line was scored in the layer with a dissecting needle, 17 cm from the bottom edge. By means of Drummond disposable pipettes, 5 μl (5 μg) of each reference compound was applied to a spot, 2 cm from the bottom edge. The plates were developed without prior activation. Development in the butanol system took 4$\frac{3}{4}$ h; in the other two solvent systems, about 1 h.

The zones were located in short-wave (254 mμ) UV light, after the solvent had evaporated from the plates. As little as 0.25 μg (*ca.* 1 nmole) of any purine could be detected in the dark (Chromato-Vue Cabinet. Ultra-Violet Products, Inc., San Gabriel, Calif.) by the quenching of the background fluorescence. The location of the

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

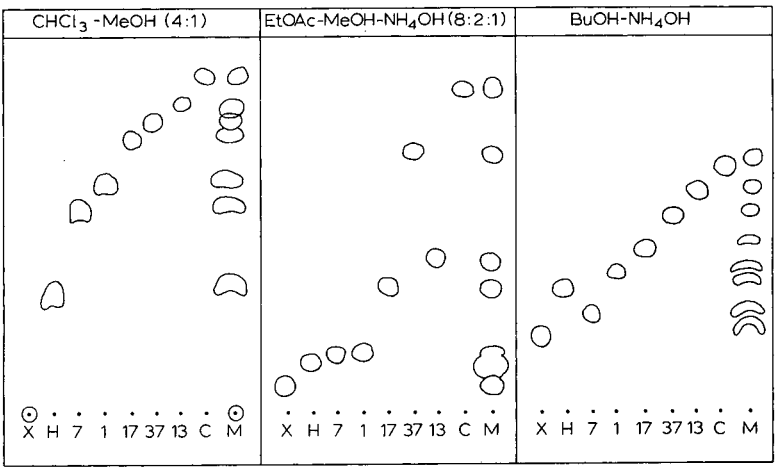

Fig. 1. Thin-layer chromatograms of xanthine derivatives in three solvent systems. Abbreviations, see under *Reference compounds*; M = mixture.

zones was marked with a dissecting needle. A permanent record was prepared by copying the chromatogram, inverted on the glass plate of a photocopier (A. B. Dick 675, A. B. Dick Company, Chicago, Ill.).

Results

As can be seen from Fig. 1, the chloroform–methanol system separates the more polar compounds better than the less polar ones. The reverse is true of the ethyl acetate-containing solvent system. Butanol–ammonium hydroxide, although much slower, has the advantage that a mixture (M) of all eight compounds can be neatly resolved at once. The mobility of hypoxanthine in the last system is greater than that of 7-methylxanthine, but lower in the first two systems. The order of mobilities for theobromine and theophylline is also reversed in the ethyl acetate-containing solvent system.

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1 R. H. KURTZMAN, JR. AND S. SCHWIMMER, *Experientia*, 26 (1971) in press.
2 G. PATAKI, *Adv. Chromatogr.*, 7 (1968) 47.
3 J. E. CIARDI AND E. P. ANDERSON, *Anal. Biochem.*, 22 (1968) 398.
4 H. DESCIMON, *Bull. Soc. Chim. Biol.*, 49 (1967) 452.
5 C. FRANZKE, K. S. GRUNERT AND H. GRIEHL, *Z. Lebensm. Unters. Forsch.*, 139 (1969) 85.
6 L. KRAUS AND E. DUMONT, *J. Chromatogr.*, 48 (1970) 96.
7 E. DUMONT AND L. KRAUS, *J. Chromatogr.*, 48 (1970) 106.
8 F. LUEDY-TENGER, *Pharm. Acta Helv.*, 45 (1970) 254.
9 P. J. ORSULAK, W. HAAB AND M. D. APPLETON, *Anal. Biochem.*, 23 (1968) 156.
10 V. M. PECHENNIKOV, A. Z. KNIZHNIK AND P. L. SENOV, *Farm. Zh. (Kiev)*, 23 (1968) 41.
11 M. RINK AND A. GEHL, *J. Chromatogr.*, 21 (1966) 143.
12 U. M. SENANAYAKE AND R. O. B. WIJESEKERA, *J. Chromatogr.*, 32 (1968) 75.
13 M. STUCHLIK, I. CSIBA AND L. KRASNEC, *Cesk. Farm.*, 18 (1969) 91.
14 R. N. WARREN, *J. Chromatogr.*, 40 (1969) 468.

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CHROM. 5397

A chromatogram staining technique useful for 3β-hydroxy-5-ene steroids and certain steroid drugs

Steroids containing the 3β-hydroxy-5-ene grouping are of some general interest, because they include starting materials and intermediates for many industrial steroid syntheses[1], the intermediates of steroid biosyntheses[2,3], and they are produced in great variety and relatively large quantity by the human foetus[4,5]. They can be detected on thin-layer chromatograms by procedures such as spraying and heating with phosphomolybdic acid, or with reagents containing various strong acids[6,7]. Such methods have low specificity, and in the case of the strong acid spray reagents, their corrosive nature causes handling difficulties. A convenient technique is now described which enables 3β-hydroxy-5-ene steroids to be detected with considerable specificity, and with sensitivity similar to that of many less specific or less convenient methods.

The technique consists of exposing the thin-layer chromatogram to hydrochloric acid vapour. In the case of silica gel layers, regions where 3β-hydroxy-5-ene steroids are present rapidly become coloured (generally pink or violet) and at first show little or no fluorescence in UV light (366 nm or 254 nm). The initial colour fades over a period of many minutes (generally going grey and finally pale yellow), and as the visible colour fades the region becomes fluorescent in UV light (366 nm and 254 nm). The yellow fluorescence in shortwave UV (254 nm) can usually be seen for several days. A few other types of steroid give colour or fluorescent reactions (which are easily distinguished from those of the 3β-hydroxy-5-enes) and the technique is useful for detecting certain steroid drugs. The technique is potentially applicable to other adsorbents (*e.g.* alumina) and some colour and fluorescence changes can even be produced on paper. However, the changes are not identical with those on silica gel, and are generally less useful (less sensitive and less easily reproduced). The following applies to silica gel layers.

The conditions for the reaction are relatively uncritical. The chromatogram may be at room temperature (22°) or at 70°, and may be exposed to the acid vapour for 5 or 15 min, giving, in most cases, similar results. Nevertheless, some standardisation is desirable, particularly if negative results are to be relied upon as evidence for the absence of compounds expected to react.

The following scheme has proved very satisfactory for 250-μ-thick layers of silica gel $HF_{254+366}$ (E. Merck, Darmstadt, G.F.R.). The plate is removed from the solvent system in which it was developed, and the solvent is allowed to evaporate. The plate is first examined in shortwave UV light (254 nm), when 3-oxo-4-ene steroids, and other compounds with suitable chromophores, appear dark against the yellow-green fluorescence of the indicator dye present in the absorbent, and their locations are marked. The plate is next examined in longwave UV light (366 nm) and a variety of saturated and unsaturated steroids, including 3β-hydroxy-5-enes, can be seen as light regions against the light blue background and their positions marked. The plate is then warmed to 40° and put into a glass tank containing a little concentrated hydrochloric acid (3 ml per l tank capacity: hydrochloric acid specific gravity 1.18 g/ml). The plate is allowed to stand in the tank for 10 min, is then removed, coloured zones are marked and the plate is examined under shortwave and longwave UV light.

TABLE I

COMPOUNDS THAT SHOWED COLOUR AND FLUORESCENCE REACTIONS DIFFERENT FROM THE 3β-HYDROXY-5-ENE COMPOUNDS

Compound	*A distinguishing feature of its reaction*
17β-Acetoxy-19-nor-17α-pregn-4-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
Androst-5-en-17-on-3β-yl sulphate (Na or NH_4 salt)	Appearance in UV
3β,17β-Diacetoxy-19-nor-17α-pregn-4-en-20-yne	Orange-red fluorescence in UV immediately after acid treatment
3-Hydroxyoestra-1,3,5(10)-trien-17-one	Slight reaction; yellow colour
17β-Hydroxy-18-methyl-19-nor-17α-pregn-4-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
17β-Hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
17β-Hydroxy-19-nor-5α,17α-pregn-20-yn-3-one	Transient pale yellow colour
3-Methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17β-ol	Fluorescent in UV immediately after acid treatment
3-Methoxyoestra-1,3,5(10)-trien-17β-ol	Slight yellow colour
3-Methoxyoestra-1,3,5(10)-trien-17-one	Very slight yellow colour
19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol	Fluorescent in UV immediately after acid treatment
Oestra-1,3,5(10)-triene-3,17β-diol	Yellow colour
Oestra-1,3,5(10)-triene-3,16α,17β-triol	Pink, fading rapidly; little fluorescence in UV
9β,10α-Pregna-4,6-diene-3,20-dione	No colour; yellow fluorescence in UV
Pregn-5-en-20-on-3β-yl sulphate (Na or NH_4 salt)	Appearance in UV

The yellow-green fluorescence normally given under shortwave UV by the indicator dye present in the absorbent is no longer visible, so the background is dark in shortwave UV, but it is still light in longwave UV. Any fluorescent spots are noted. The plate is re-examined after 10–20 min, and again after 1–2 h.

The sequence of colour and fluorescence changes regarded as characteristic of 3β-hydroxy-5-enes is as follows. A colour, generally pink, develops within a minute or two of exposure to the acid vapour. The colour depends upon the compound, ranging from orange-pink to purple-pink for the compounds tested, except for 3β,11β,17α,21-tetrahydroxypregn-5-en-20-one, which gave a blue colour. The time from exposure to acid vapour required for the colour to become easily visible depends upon the amount of material present, and was usually 1–2 min for 10–2 μg of steroid per cm^2. Immediately after the standard 10 min exposure to acid vapour, the coloured spots show little or no fluorescence in shortwave or longwave UV light. The coloured spots can be seen to be fading a few minutes after removal from the acid vapour, and when examined after 10–20 min are seen to be greyer (visible light), slightly fluorescent in longwave UV light, and weakly but clearly fluorescent (yellow) in shortwave UV light. After 1–2 h the coloured spots have largely faded, and may be yellow, yellow-grey or a pale brown colour. When less than about 2 μg of steroid per cm^2 is present, these faded colours generally cannot be seen. In shortwave UV light, the faded spots are fluorescent (yellow) against the dark background; a similar fluorescence in longwave UV often appears somewhat different against the light background and may be less distinct. A slight colour (esp. yellow) in visible light and yellow fluorescence in shortwave UV light are usually still present after several days.

The smallest amounts of steroid that can be clearly distinguished are in the

region of 1 $\mu g/cm^2$, *e.g.* about 0.6 $\mu g/cm^2$ for 3β-hydroxycholest-5-ene, about 0.8 $\mu g/cm^2$ for 3β-hydroxypregn-5-en-20-one, about 1 $\mu g/cm^2$ for 3β-hydroxyandrost-5-en-17-one, and about 1.5 $\mu g/cm^2$ for 3β,17α-dihydroxypregn-5-en-20-one.

Some steroids other than 3β-hydroxy-5-enes give coloured and/or fluorescent products when treated as described above (see Table I). A detailed description of the changes for each compound is unnecessary. The compounds include various oestrogens, and it is seen that a free hydroxyl group at the 3 position is not essential. The striking reactions of 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3β,17β-diol (ethynyloestradiol) and its 3-methyl ether (mestranol) no doubt involve participation of the 17α-ethynyl group. Several compounds containing this group gave marked reactions, while the corresponding de-ethynyl compounds did not. However, the one 17α-ethynyl compound tested which had a saturated steroid ring system, namely 17β-hydroxy-19-nor-5α,17α-pregn-20-yn-3-one, gave only a transient yellow colour, so the production of the more striking colour changes in this series involved reactive groups in the A ring as well as the 17α-ethynyl group. In this connection, 3β,17β-diacetoxy-19-nor-17α-pregn-4-en-20-yne (ethynodiol diacetate) was interesting because it contained the acetate ester of the allylic alcohol derivable from a 3β-hydroxy-5-ene (by migration of the double bond from 5 to 4) and a 17α-ethynyl group. In fact it gave a spectacular sequence of colour and fluorescence changes, and was detectable down to about 0.4 $\mu g/cm^2$. Reaction of 9β,10α-pregna-4,6-diene-3,20-dione (dydrogesterone) to give a colourless but fluorescent product was interesting because the other three 3-oxo-4,6-dienes (chlormadinone acetate, megestrol acetate and medrogestone) and the other 9β,10α compound (retrotestosterone) tested did not behave similarly.

Steroids found not to give any clear colour or fluorescence reaction under the standard test conditions include most of the naturally occurring structural types other than the 3β-hydroxy-5-enes, and oestrogens. They also include various drugs and synthetic intermediates. In many cases, these compounds probably remained unchanged. This was tested in selected cases as follows. The steroid was applied to a chromatogram, and exposed to acid vapour under the standardised conditions before the chromatogram was developed with solvent. The chromatogram was allowed to stand for several minutes (about 20 min) in the draught of a fume-hood and a further specimen of the same steroid was then applied in an adjacent lane, the chromatogram was immediately developed with solvent, and subsequently sprayed with dinitrophenylhydrazine reagent (saturated 2,4-dinitrophenylhydrazine in propan-2-ol, freshly acidified with concentrated hydrochloric acid, 0.2 ml per 20 ml). The spots obtained from the steroid exposed to acid vapour and that applied after acid treatment were then compared. In this way it appeared that 17β-hydroxyandrost-4-en-3-one, its propionate, the corresponding *retro* compound (17β-hydroxy-9β,10α-androst-4-en-3-one), and the related reduced compounds 17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5α-androstan-17-one, and 3α-hydroxy-5β-androstan-17-one remained substantially unchanged. So too did 21-hydroxypregn-4-ene-3,20-dione, its acetate, and 17α-acetoxy-6-methylpregna-4,6-diene-3,20-dione. However, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (cortisol) and 17α,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) evidently underwent some decomposition. It is interesting to note that the ester groups of a 21-acetate (primary hydroxyl), a 17β-propionate (secondary hydroxyl), and a 17α-acetate (a tertiary hydroxyl in this case), were not appreciably hydrolysed. Thus, although 3β-acetoxypregn-5-en-20-one gave a colour and fluo-

rescence reaction closely similar to 3β-hydroxypregn-5-en-20-one, it is unlikely that reaction of the acetate depended upon formation of the free 3β-hydroxy compound sa an intermediate. Pregn-5-en-20-on-3β-yl sulphate and androst-5-en-17-on-3β-yl sulphate (as sodium or ammonium salts in either case) gave reactions similar to but distinguishable from the corresponding free steroids.

An advantage of the staining technique described is that it allows many common spray reagents to be used subsequently on the same plate. In this way much information is obtained for little expenditure of laboratory time or valuable specimens.

The present findings may be contrasted with the report (ref. 7, p. 27) that when plates are sprayed intensively with concentrated hydrochloric acid and heated at 80–100° for 5 min, a blue colour is obtained from 3,7-dihydroxy-5-ene steroids, but no colour is obtained with other 3-hydroxy-5-ene or 3-hydroxy-4-ene steroids. The opportunity has not yet arisen to test any 3,7-dihydroxy-5-enes using the new technique.

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1 N. APPLEZWEIG, *Steroid Drugs*, McGraw-Hill, New York, 1962, pp. 53–86.
2 G. POPJAK AND J. W. CORNFORTH, *Adv. Enzymol.*, 22 (1960) 281.
3 R. I. DORFMAN AND F. UNGAR, *Metabolism of steroid hormones*, Academic Press, New York, 1965.
4 F. L. MITCHELL, *Vitam. Horm.*, 25 (1967) 191.
5 O. A. JÄNNE AND R. K. VIHKO, *Eur. J. Biochem.*, 17 (1970) 134.
6 R. NEHER, *Steroid Chromatography*, Elsevier, Amsterdam, 1964, pp. 258–266.
7 B. P. LISBOA, *Methods Enzymol.*, 15 (1969) 19.

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A reagent for the non-destructive location of steroids and some other lipophilic materials on silica gel thin-layer chromatograms

There is a frequent need to locate UV non-absorbing steroids on silica gel thin-layer chromatograms by means which conserve the detected material for further investigation. It has been found that a spray reagent prepared by diluting 1 ml of a stock solution of primuline (0.1 g in 100 ml water) with 100 ml of a mixture of acetone and water (4:1) has wide application for this purpose. Primuline (Michrome No. 64), supplied by Edward Gurr Ltd., London SW14, is described in ref. 1. Merck Kieselgel precoated plates and calcium sulphate bound plates prepared in the laboratory with Machery Nagel Kieselgel proved satisfactory. Plates prepared with Machery Nagel Kieselgel bound with starch showed reduced sensitivity of detection. The primuline reagent was ineffective on partition chromatograms developed on Antec cellulose thin-layer plates. Developed silica gel chromatograms, free of developing solvent are lightly sprayed and viewed while still damp under a high pressure mercury vapour lamp with maximum output at 365 mμ (230–240 V, 125 W, Osram high pressure mercury vapour lamp, MBW/U, manufactured by GEC). A variety of both UV absorbing and UV non-absorbing steroids are visualized as pale yellow or pale blue fluorescent spots on a weakly fluorescent background. Listed below are some representative steroids which have been detected, with their approximate sensitivity limits on Merck Kieselgel precoated plates given in parentheses: pregnenolone, dehydroepiandrosterone, 5α-pregnane-3,20-dione, androsterone, 17α-hydroxypregnenolone, 3β,17β-dihydroxy-5α-androstane (0.5–1 μg); progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, 11-deoxycorticosterone, corticosterone, cortisone (1–2 μg); cortisol, oestrone, oestradiol, oestriol (2–5 μg). Oestrogen 3-methyl ethers and a number of steroid formates and acetates have also been detected satisfactorily. Sensitivity of detection diminishes as the plates dry out but may be restored by lightly spraying with acetone–water (4:1). Plates with incorporated UV-254 indicator serve as well as those without as this indicator shows no fluorescence at the longer wavelength utilized.

As an alternative procedure, plates may be predipped in the reagent and allowed to dry thoroughly before steroids are applied and the chromatograms developed. Although the sensitivity is lower than that given by the spray procedure the method has occasional advantages and sensitivity may be improved if necessary by spraying with the acetone–water mixture as described.

Preliminary experiments in which the primuline spray has been used successfully to locate: cholesterol, cholesterol oleate, 1-monopalmitin, diolein, triolein, oleic acid and the organochlorines 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT); 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (DDE); 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDD); bis-(*p*-chlorophenyl)acetic acid (DDA) and DDA methyl ester suggest that this reagent may have widespread application for the non-destructive location of other lipophilic materials on silica gel thin-layer plates.

This work forms part of the programme of the Marine Laboratory of the Department of Agriculture and Fisheries for Scotland.

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1 E. GURR, *Encyclopaedia of Microscopic Stains*, Leonard Hill, London, 1960, p. 339.

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Thin-layer chromatography of Thalictrum species alkaloids

Plants belonging to the *Thalictrum* L. species contain numerous basic components representing various types of structure. The identified compounds belong to the following groups: (I) isoquinoline alkaloids, 2 substances; (II) benzylisoquinoline alkaloids, 2 substances; (III) aporphine alkaloids, 8 substances; (IV) bisbenzylisoquinoline alkaloids, 18 substances; (V) aporphinobenzylisoquinoline alkaloids, 4 substances; (VI) hexahydrotriphenilidine alkaloids, 2 substances; (VII) protoberberine alkaloids, 9 substances, and (VIII) glucoalkaloids, at least 46 substances altogether.

The majority of the alkaloids consists of tertiary compounds, the rest is composed of quaternary substances; the separation of both types takes place during extraction or purification of the extracts.

In the course of investigating the compounds contained in the roots of *Thalictrum minus* L. var. *elatum* Koch. a fraction of tertiary products was obtained which showed 11 spots on the chromatograms. The fraction yielded two products identified as aporphinobenzylisoquinoline alkaloids: a new alkaloid thalmineline, the structure of which could be fully elucidated[5], and another product designated 'compound C'[3].

The eleven-spot fraction of quaternary bases yielded berberine, magnoflorine, jatrorrhizine, and thaliphendine[3] as pure substances.

The initial tests on the constituents of the above-mentioned plant were carried out by thin-layer chromatography (TLC), the details of the procedure being worked out with standard substances, including 16 tertiary bases and 6 quaternary ones, obtained from various research centres.

Experimental

The plates covered with Silica Gel G or Aluminium Oxide according to Stahl were activated at 120° for 2 h.

The following systems were found in the course of numerous experiments as the most convenient ones for the separation of tertiary alkaloids.

For plates covered with Silica Gel G:

System A, benzene–chloroform–isopropanol–ammonia (90:90:16:20).

System B, benzene–ethyl acetate–methanol (75:75:100); a modification of PFEIFER's[4] system.

System C, benzene–chloroform–methanol–ethyl acetate (2:7:3:1); a system developed by PFEIFER[4].

For plates covered with Aluminium Oxide G:

System D, benzene–chloroform (1:9) (ref. 1).

System E, ether.

The quaternary alkaloids were chromatographed on Silica Gel G according to Stahl in the solvent chloroform–methanol–ammonia (75:30:5) (system F). Berberine, palmatine, and columbamine which cannot be separated with system F were chromatographed on the same sorbent in system G which consisted of chloroform–methanol–pyridine–monoethylamine (50% aqueous solution) (100:40:10:5) (ref. 2).

The following detection reagents were employed: (a) Dragendorff's reagent;

TABLE I

R_F VALUES OF SELECTED TERTIARY ALKALOIDS FOUND IN THE THALICTRUM SPECIES

Designation	*System*				
	A	*B*	*C*	*D*	*E*
Thaliphendlerine (II)	0.43	0.69	0.77	0.10	0.37
Laudanosine[a] (II)	0.97	0.49	0.72	0.78	0.71
Isocorydine (III)	0.95	0.72	0.85	0.89	0.89
Glaucine (III)	0.98	0.69	0.89	0.87	0.85
Obamegine (IV)	0.35	0.23	0.48	0.10	0.08
Berbamine (IV)	0.48	0.29	0.56	0.10	0.22
Hernandesine (IV)	0.79	0.45	0.73	0.65	0.00
Thalidasine (IV)	0.80	0.78	0.89	0.55	0.80
Thalidesine (IV)	0.50	0.00	0.63	0.00	0.00
Thalmetine (IV)	0.60	0.79	0.85	0.00	0.00
O-Methylthalmetine (IV)	0.70	0.83	0.68	0.69	0.15
Thalmelatine (V)	0.55	0.30	0.59	0.09	0.09
Thalicarpine (V)	0.70	0.30	0.67	0.70	0.70
Thalmineline (V)	0.45	0.71	0.81	0.21	0.10
Tetrahydrothalifendine (VII)	0.50	0.43	0.24	0.00	0.00
Veronamine (VIII)	0.07	0.54	0.51	0.00	0.00

[a] Has not been discovered in Thalictrum sp. up to the present.

(b) diazotized *p*-nitraniline (aromatic hydroxy groups); (c) Labat reagent (dioxymethylene groups).

The alkaloids were spotted on the plates in 20 μg amounts as chloroform or methanolic solutions.

The chromatograms were developed for 12 cm. After drying, they were inspected in UV light and sprayed with the Dragendorff reagent, Labat reagent, and with diazotized *p*-nitraniline. The R_F values are reported in Tables I and II.

The results of two-dimensional chromatography are shown in Fig. 1.

Discussion

Tables I and II report all the R_F values found for the alkaloids studied. Each of the systems recommended for the tertiary alkaloids may be successfully used for the separation of a mixture consisting of several compounds, *e.g.* as a fraction isolated from plant material.

TABLE II

R_F VALUES OF SOME QUATERNARY ALKALOIDS FOUND IN THE THALICTRUM SPECIES

Designation	R_F *value in system F*	*–OH*	$-CH_2-O-CH_2-$
Berberine	0.75		+
Palmatine	0.75		
Columbamine	0.75	+	
Magnoflorine	0.43	+	
Jatrorrhizine	0.65	+	
Thaliphendine	0.88	+	+

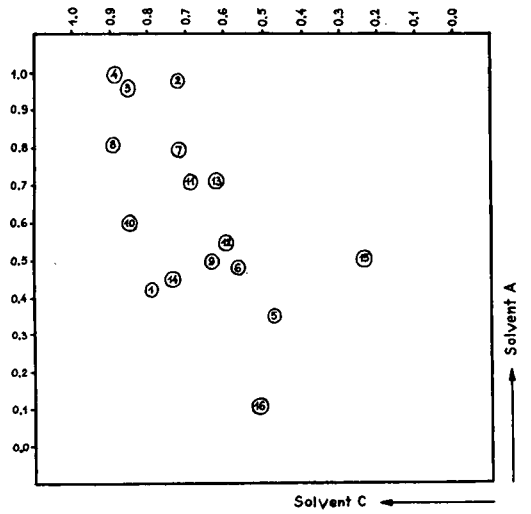

Fig. 1. Two-dimensional chromatogram of a mixture of tertiary alkaloids. 1 = Thaliphendlerine; 2 = laudanosine; 3 = isocozydine; 4 = glaucine; 5 = obamegine; 6 = barbamine; 7 = hernandesine; 8 = thalidasine; 9 = thalidesine; 10 = thalmetine; 11 = O-methylthalmetine; 12 = thalmelatine; 13 = thalicarpine; 14 = thalmineline; 15 = tetrahydrothalifendine; 16 = veronamine. Solvent systems: (A) benzene–chloroform–isopropanol–ammonia (90:90:16:20); (C) benzene–chloroform–methanol–ethyl acetate (2:7:3:1).

In practice, phenolic bases and non-phenolic ones were chromatographed separately.

Application of two-dimensional chromatography with systems A and C, or A and B, permits the separation of 16 alkaloids, as shown in Fig. 1.

In our experiments on the isolation of the alkaloids from a fraction obtained from *Th. minus.* var. *elatum* Koch. some bases were found which had identical R_F values to standard substances, sometimes even in two solvent systems. Further tests, however, did not confirm their identity. For full chromatographic identification it is thus necessary to test all five of the recommended systems.

System F, developed for the quaternary alkaloids, is unsuitable for berberine, palmatine, and columbamine which, in this system, have identical R_F values.

System F may, however, be used successfully for the separation of the majority of the alkaloids present in various genera of the *Thalictrum* species. It was tested for *Th. elegans* Wall. (5 spots); *Th. fendleri* Engelm. (11 spots); *Th. minus* var. *elatum* Koch. (11 spots); *Th. minus* var. *nanus* (10 spots); *Th. pedunculatum* Edgew. (11 spots); *Th. rhinchocarpum* Dill. et Rich. (9 spots); and *Th. squarosum* (9 spots). The largest spots were always those of magnoflorine and berberine.

Berberine, palmatine, and columbamine were identified in system G, the alkaloids showing R_F values of 0.48, 0.40, and 0.15, respectively.

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1 T. CIESZYŃSKI, *Doctoral Thesis*, Poznań, 1963.
2 I. HORN AND K. STEFFEN, *Pharm. Zeit.*, 26 (1968) 945.
3 T. KANIEWSKA, *Doctoral Thesis*, Warszawa, 1970.
4 S. PFEIFER AND H. DÖHNERT, *Pharmazie*, 23 (1968) 585.
5 J. REISCH, H. ALFEN, T. KANIEWSKA AND B. BORKOWSKI, *Tetrahedron Lett.*, 24 (1970) 2113.

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The separation of the photochemical isomers of ergosterol by thin-layer chromatography

The separation of the photochemical isomers of ergosterol presents a difficult experimental problem. The investigations of WINDAUS[1], VELLUZ[2] and HAVINGA[3] have shown that UV irradiation of ergosterol at a temperature below 20° gives rise to five isomers: prévitamin D, tachysterol$_2$, lumisterol$_2$, toxisterol$_I$ and toxisterol$_{II}$. If the irradiation temperature is higher than 20° three additional isomers are formed: vitamin D$_2$, suprasterol$_I$ and suprasterol$_{II}$.

Many known chromatographic methods have been used in the study of the ergosterol isomers[4–7] and recently we have separated vitamin D$_2$ and the suprasterols[8].

Experimental

Preparation of solutions. Solutions of ergosterol in ethyl ether (5 g/l) were irradiated in quartz cells by two Mazda TG16 germicide lamps at a wavelength of 2537 Å and at a temperature below 20°. Two ergosterol solutions containing different

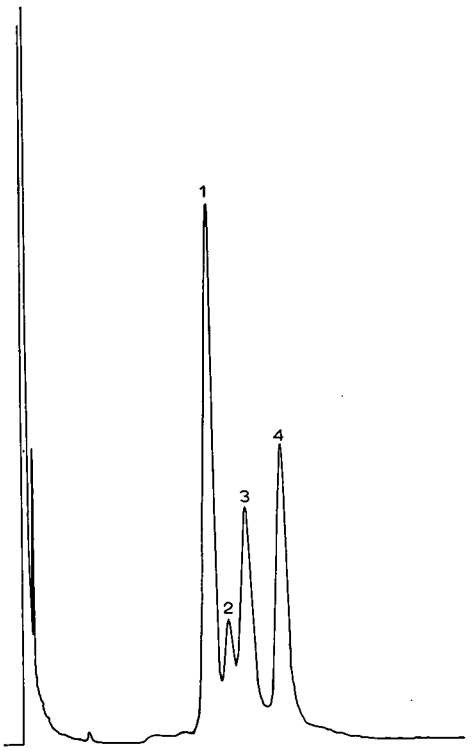

Fig. 1. Gas phase chromatography of the photochemical isomers of ergosterol. 1 = Pyrocalciferol$_2$ + lumisterol$_2$; 2 = isopyrocalciferol$_2$; 3 = ergosterol; 4 = tachysterol$_2$. Column: 3% JXR.

isomer concentrations are obtained after irradiations of 2.30 h and 15 h duration. Since the lumisterol$_2$ concentration is very small for the wavelength used, a 1 g/l solution of lumisterol$_2$ in ethyl ether is added to the two solutions of irradiated ergosterol. The purity of the solution is checked by gas phase chromatography on an apolar stationary phase[11] (Fig. 1).

Preparation of the plates. Three types of plates were used. Type E aluminium oxide plates (Merck ref. 5713), thickness 250 μm. Type T aluminium oxide plates (Merck ref. 1065), thickness 1 mm. Kieselgel plates (Merck ref. 5715), thickness 250 μm.

The plates are activated for 3 h at 115° and a 15 mm wide band is scraped off the layer at each side in order to reduce the edge effect.

Solvent systems. Tests were carried out on some twenty solvents or pairs of solvents.

Four solvent pairs were chosen:

S1, methylene chloride–ethyl acetate (14:4) for the type T Al_2O_3 plates.

S2, carbon tetrachloride–acetone (10:1) for the type T Al_2O_3 plates and the Kieselgel plates.

S3, benzene–acetone (9:1) for the type E Al_2O_3 plates and the Kieselgel plates.

S4, benzene–ethyl acetate (10:2.5) for the type E Al_2O_3 plates.

Chromatography. The solutions were deposited 30 mm from the lower edge of the plates, in the form of spots containing between 10 and 20 μg of product. The chromatographic tanks did not contain any filter paper for saturation. The internal temperature of the development tanks was 20°. This factor plays a part, according to SINGH[12] and according to our own tests.

Detection. A 20% solution of antimony trichloride in chloroform is sprayed on to the plates after development. A 1-min heating with hot air is sufficient to show up the different isomers with their characteristic colours (Table I).

TABLE I

COLOURS OF THE ERGOSTEROL ISOMERS AFTER DETECTION ON THE THIN-LAYER PLATES

Isomer	*Colour*
Ergosterol	Blue
Lumisterol$_2$	Raspberry pink
Tachysterol$_2$	Dark brown
Prévitamin D	Light brown
Vitamin D_2	Light brown
Suprasterol$_I$	
Suprasterol$_{II}$	

Results

Table II shows the R_F values of the different isomers, in various solvent systems, of an ergosterol solution irradiated for 2.30 h with added lumisterol$_2$ and vitamin D_2 in ethyl ether.

The two irradiated solutions, with addition of the lumisterol$_2$ solution, were

TABLE II

R_F VALUES OF ERGOSTEROL STEREOISOMERS

	Type T Al_2O_3 plates		*Type E Al_2O_3 plates*		*Kieselgel plates*	
	S1	*S2*	*S3*	*S4*	*S2*	*S3*
Ergosterol	0.38	0.34	0.17	0.38	0.45	0.49
Vitamin D_2	0.51	0.47	0.27	0.51	0.59	0.60
Tachysterol$_2$	0.63	0.55	0.33	0.58	0.62	0.62
Lumisterol$_2$	0.68	0.62	0.52	0.67	0.70	0.68
Provitamin D	0.74	0.71	0.57	0.72	0.78	0.75

then applied in the form of lines rather than spots on the thin layer. A capillary pipette is used for this.

Kieselgel plates, 250 μm thick, were used. Using the solvent mixture carbon tetrachloride–acetone (10:1) it was possible, after development, to scrape the bands formed off the plates, elute them with ethyl ether and, after filtration on pre-washed Durieux paper, to obtain the UV spectra of the main isomers (see Fig. 2). These spectra are identical to those given by SHAW[13].

The reproducibility of the results was checked by running several tests. Using 2 mm thick layers it was possible to isolate enough product to use gas phase chromatography and obtain the retention times characteristic for each isomer.

Discussion

Using Kieselgel plates the separation of vitamin D_2 from tachysterol$_2$ is difficult

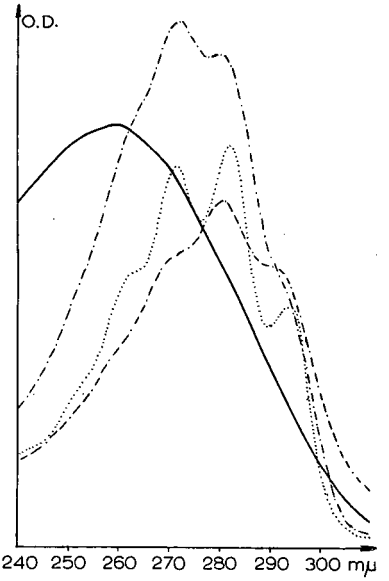

Fig. 2. UV absorption spectra obtained by elution of the bands formed on 250 μm plates. Ergosterol solution irradiated 2.30 h. (———) Prévitamin D; (—·—) lumisterol$_2$; (· · · · · ·) ergosterol; (· — — ·) tachysterol$_2$.

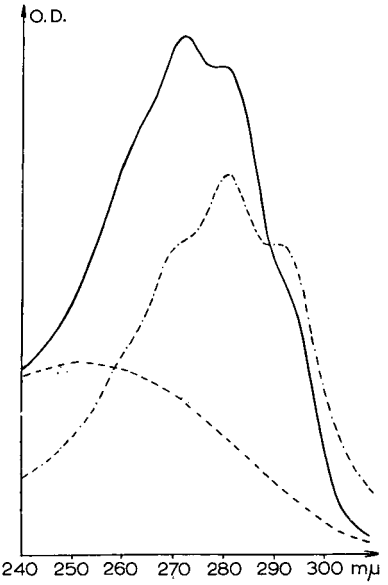

Fig. 3. UV absorption spectra obtained by elution of the bands formed on 250 μm plates. Ergosterol solution irradiated 15 h. (———) Lumisterol$_2$; (· — — ·) tachysterol$_2$; (— — —) toxisterol$_2$A or oxidation product.

whereas lumisterol$_2$ is completely separated from prévitamin D. With type E aluminium oxide, the results are the opposite of those obtained with Kieselgel. The best results for the separation of the five main isomers are in fact obtained with type T aluminium oxide. If the irradiations are carried out at temperatures below 20°, the transition from the prévitamin to vitamin D$_2$ is excluded and the four isomers formed may be studied on Kieselgel.

The UV spectra obtained on products separated by chromatography on a 250 μm layer, instead of 2 mm, are given for ergosterol solutions irradiated for 2.30 h (Fig. 2). The spectra obtained for the 15 h irradiation times, still with addition of a lumisterol$_2$ solution, are given in Fig. 3.

Two differences can be seen in the spectra of Figs. 2 and 3. Firstly, ergosterol does not appear in Fig. 3 since it now exists only in a very small quantity; this is confirmed by gas phase chromatography. Secondly, the prévitamin spectrum is different, the absorption maximum being displaced towards the short wavelengths.

The method giving the UV spectra by elution of the bands formed on thin 250 μm layers also proved suitable for the study of the photochemical isomers of 7-dehydrocholesterol.

The R_F values for 7-dehydrocholesterol, the prévitamin and tachysterol$_3$ are identical with those of the ergosterol isomers, using Kieselgel layers and the solvent mixtures benzene–acetone (9:1) and carbon tetrachloride–acetone (10:1).

Conclusions

In this work it was possible to use thin-layer chromatography and absorption spectra of four isomers were obtained by elution of the bands formed on 250 μm analytical plates.

This chromatographic technique, which has only occasionally been applied to the study of ergosterol isomers[5,6,9,10], seems to be very well suited for this problem.

We wish to thank M. VERMEUREN of the Prochimex Company, France, and the Philips-Duphar Company, for gifts of lumisterol$_2$ samples.

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1 A. WINDAUS, *Les Vitamines et les Hormones*, Gauthier-Villars, Paris, 1938, p. 91.
2 L. VELLUZ, *Conférence à l'Université de Leyde (24-4-1959)*.
3 E. HAVINGA, *Chimia (Aarau)*, 16, No. 5 (1962) 145–151.
4 E. KODICEK, *Biochem. J.*, 57 (1954) XII, XIII.
5 A. W. NORMAN-DE LUCA, *Anal. Chem.*, 35 (1963) 1247.
6 T. KOBAYASHI, *J. Vitaminol.*, 13 (1967) 258.
7 J. W. COPIUS PEEREBOOM, J. B. ROOS AND H. W. BEEKES, *J. Chromatogr.*, 5 (1961) 500.
8 R. MERMET-BOUVIER, *Bull. Soc. Chim. France*, 8/9 (1970) 174.
9 H. A. BOGOLOVSKI, *Med. Prom. SSSR.* 19 (1965) 41.
10 G. M. SANDERS AND E. HAVINGA, *Rec. Trav. Chim.*, 83 (1964) 665.
11 R. MERMET-BOUVIER, *Séparations des Isomères Photochimiques de l'Ergostérol par Chromatographie sur Phase Gazeuse*, to be published.
12 E. J. SINGH, *J. Chromatogr. Sci.*, 8 (1970) 162.
13 W. H. SHAW, *Analyst*, 82 (1957) 2.

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CHROM. 5340

Application of thin-layer chromatography to the purification and characterization of some methyl glucosides

The recent elucidation of the structure of the monomycolylglucoses of some corynebacteria and mycobacteria[1] involved synthesis of a number of methyl glucosides. Several methods were investigated to prepare pure samples of these compounds. The most satisfactory and rapid procedure employed preparative thin-layer chromatographic (TLC) systems. This note describes the application of these systems to the purification of α- and β-methyl tetra-O-methylglucosides and the anomers of methyl 2,3,4-tri-O-methylglucoside. The characterization of these compounds by TLC and other methods is also described.

Experimental

Preparation of the methyl glucosides. Two precursors of methyl glucosides were used, *viz.* methyl α-D-glucoside and isomaltose (both obtained from Koch-Light). The methylation of methyl α-D-glucoside was based on the procedure of PEAT[2]. Methyl α-D-glucoside (350 mg) was dissolved in 3 ml of distilled anhydrous methanol. To this were added 1.26 g of freshly prepared Ag_2O and 1.55 g of CH_3I. The mixture was refluxed for short spells over a 4-h period and allowed to cool for the intermittent times. At the end of this period further portions of CH_3I and Ag_2O were added. The mixture was stirred at room temperature for 5 h and finally refluxed while stirring for 15 min. The solution was filtered and the Ag_2O washed with warm methanol. The filtrates were concentrated, extracted with distilled diethyl ether and the extract was dried over anhydrous sodium sulphate, filtered and concentrated to give a clear viscous syrup.

Methylation of isomaltose was carried out in two ways. The first employed the method of KUHN *et al.*[3] as follows: 20 mg of isomaltose, 2 ml of dimethylformamide, 2 ml of CH_3I and 2 g of Ag_2O were placed in a test tube and shaken vigorously for 24 h. Similar amounts of Ag_2O and CH_3I were then added and the solution shaken for a further 24 h. The mixture was filtered and the Ag_2O was washed with $CHCl_3$. The filtrates were concentrated and equal volumes of water and benzene added and the mixture shaken. The upper benzene layer was washed several times with water and finally concentrated to yield a yellow syrup which was dried *in vacuo* over P_2O_5.

The methylation procedure of BRIMACOMBE *et al.*[4] was also applied to isomaltose as follows: isomaltose (15 mg) was dissolved in 1 ml of dimethylformamide. Sodium hydride (50 mg) was then added, followed by CH_3I (1 ml). The solution was stirred for 15 min at room temperature and a further 1 ml of dimethylformamide was added. After 2-h stirring at room temperature the mixture was added to chloroform–water (1:1). The chloroform layer was repeatedly washed with water, finally dried over sodium sulphate and evaporated to dryness.

Methanolysis and hydrolysis. The permethylated isomaltose obtained by both procedures was methanolysed in a sealed tube with 3 ml of 4.6% anhydrous methanolic HCl at 105° for 18 h. The tube was opened, methanol was removed on a rotary evaporator and HCl in a desiccator over KOH. Reducing sugars were obtained from the corresponding methyl glucosides by incubating in sealed tubes at 100° with 2 *N* HCl for 2 h.

Chromatographic systems. Preparative TLC was carried out on wide plates (40 × 20 cm) of Silica Gel H (0.5 mm thick). Chloroform–methanol (47:3) (solvent A)[5] was used to obtain full resolution of the methyl glucosides with different numbers of methyl groups arising from methyl α-D-glucoside. This solvent was not satisfactory for resolving the α- and β-anomers of the methyl glucosides, in which case benzene–ethanol–water (170:47:15, upper layer)[6] (solvent B) was used. Reducing sugars were chromatographed on thin-layer plates of Silica Gel G (0.25 mm thick) in acetone–25% (w/v) aqueous ammonia–water (250:1.5:3)[7] (solvent C) or on sheets of Whatman No. 1 paper in butanol–ethanol–water (50:10:4, upper layer). Gas–liquid chromatography (GLC) was carried out on columns of 10% diethylene glycol succinate (DEGS) on Chromosorb W, 100/120 mesh, at 175° and with a nitrogen flow rate of 45 ml/min. A column of 2% neopentyl glycol succinate (NPGS) on Chromosorb W, temperature programmed at 3° per min from 120° to 250°, and with a nitrogen flow rate of 45 ml/min, was also used to indentify the α- and β-anomers of the methyl tri-O-methylglucoside.

Results and discussion

The methylated glucosides obtained from the methylation of methyl α-D-glucoside were dissolved in chloroform and applied to four preparative thin-layer plates and chromatographed in solvent A. The plates were divided into nine equal bands parallel to the line of origin and these were cut out except for two narrow strips (1.5 cm wide) on each margin of the plate. The strips were sprayed with 50% H_2SO_4 and heated at 110° for 20 min. Four compounds were present (Table I). These were eluted from the respective bands of silica gel with chloroform–methanol (2:1). The mono- and di-O-methylglucosides were not examined further. The tetra-O-methylglucoside in solvent B had an R_F value of 0.49 identical to methyl 2,3,4,6-tetra-

TABLE I

RESOLUTION OF SOME METHYL GLUCOSIDES BY TLC

Source	*R_F value*		*Relative amounts*	*Structure*
	Solvent A	*Solvent B*		
Methylated methyl α-D-glucoside	1.00		+	methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside
	0.63		++++	methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside
	0.32		++	a methyl di-O-methyl-α-D-glucopyranoside
	0.12		+	a methyl mono-O-methyl-α-D-glucopyranoside
Methylated, methanolysed isomaltose		0.62	+++	methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside
		0.49	++	methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside
		0.35	+++	methyl 2,3,4-tri-O-methyl-β-D-glucopyranoside
		0.31	++	methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside

O-methyl-α-D-glucopyranoside (a gift from Professor P. O'COLLA, University College, Galway). GLC of this material showed a retention time of 6.9 min identical to that of methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside. PC of the reducing sugar showed one spot (detected with aniline–phthalate) with an $R_{Glucose}$ value of 4.28, the same as that for 2,3,4,6-tetra-O-methylglucose. TLC of the reducing sugar in solvent C again showed one spot with an R_F value of 0.78 identical to 2,3,4,6-tetra-O-methylglucose.

In solvent B the tri-O-methyl-α-D-glucoside had an R_F value of 0.30 identical to that of methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside (a gift from Dr. G. BATHGATE, Guinness Research Laboratories, Dublin). GLC of this material on the DEGS column showed a retention time of 27.6 min identical to the methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside. PC of the reducing sugar showed an $R_{Glucose}$ value similar to that of 2,3,4-tri-O-methylglucose.

Permethylated isomaltose was methanolysed and the product applied to preparative thin-layer plates and chromatographed in solvent B. When strips from the plates were charred with 50% H_2SO_4, four bands were observed (Table I). The methyl glucosides were eluted and identified by TLC and PC as described above. GLC of the methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside showed a retention time of 5.1 min compared with 7.5 min for the α-anomer. The methyl 2,3,4-tri-O-methyl-β-D-glucopyranoside had a retention time of 19.8 min compared to 27.6 min for the α-anomer.

Solvent A has proved very effective for the resolution of mono-, di-, tri- and tetra-O-methylglucosides and solvent B can separate the anomeric forms of the methyl tri-O-methylglucosides and the methyl tetra-O-methylglucosides. A combination of these solvents is a useful adduct to GLC[8] for locating the position of fatty acids on the glucopyranose of the acylglucoses[1,9].

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1 P. J. BRENNAN, D. P. LEHANE AND D. W. THOMAS, *Eur. J. Biochem.*, 13 (1970) 117.
2 S. PEAT, *J. Chem. Soc.*, 83 (1903) 1037.
3 R. KUHN, H. TRISCHMANN AND I. LOW, *Angew. Chem.*, 67 (1955) 32.
4 J. S. BRIMACOMBE, B. D. JONES, M. STACEY AND J. J. WILLARD, *Carbohyd. Res.*, 2 (1966) 167.
5 T. S. STEWART, P. B. MENDERSHAUSEN AND C. E. BALLOU, *Biochemistry*, 7 (1968) 1843.
6 C. PROTTEY AND C. E. BALLOU, *J. Biol. Chem.*, 243 (1968) 6196.
7 P. J. STOFFYN, *J. Amer. Oil Chem. Soc.*, 43 (1966) 69.
8 G. MARTIN AND J. ASSELINEAU, *J. Chromatogr.*, 39 (1969) 322.
9 P. J. BRENNAN, M. P. FLYNN AND P. F. S. GRIFFIN, *FEBS Letters*, 8 (1970) 322.

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CHROM. 5403

Separation of porphyrin methyl esters by two-dimensional thin-layer chromatography

Qualitative[1,2], quantitative[3] and preparative[4] methods for the separation of porphyrin methyl esters by one-dimensional thin-layer chromatography (TLC) have been described. Porphyrin methyl esters with low R_F values are poorly differentiated in these systems and may be obscured by other pigments, often present in extracts from biological sources. Two-dimensional paper chromatography has been used to resolve mixtures of methyl esters of dicarboxylic porphyrins[5,6], separation being especially satisfactory with hydroxylated porphyrins. This paper describes a simple two-dimensional TLC method which has some advantages over existing techniques.

Materials and method

Solvents. Chloroform was washed three times with water and dried by shaking with anhydrous Na_2SO_4, and by filtration through a triple layer of chloroform moistened filter papers, before use. Kerosene (white) was obtained from Hopkin and Williams. All other solvents were of analytical reagent grade, except for butanone (special for chromatography), methyl acetate and propionate (laboratory reagent grade) and dichloromethane (redistilled) from British Drug Houses.

Porphyrins. Protoporphyrin IX dimethyl ester (Grade A) and coproporphyrin III tetramethyl ester were from Sigma; mesoporphyrin IX dimethyl ester from Koch-Light Laboratories. Synthetic harderoporphyrin trimethyl ester was a gift from Professor A. H. JACKSON, Department of Organic Chemistry, University of Wales, Cardiff. Uroporphyrin, hepta-, hexa-, and pentacarboxylic porphyrin methyl esters were isolated from porphyric urine. Haematoporphyrin IX dimethyl ester[7], isohaematoporphyrin IX dimethyl ester, bis-β-hydroxypropionic deuteroporphyrin IX tetramethyl ester[8], and mono-β-hydroxypropionic, monopropionic deuteroporphyrin IX tetramethyl ester were prepared from protoporphyrin IX dimethyl ester.

Chromatographic procedure. Glass plates (20 × 20 cm) were spread with silica gel (Camag D5) to a depth of 0.3 mm (Shandon Unoplan leveller with adjustable spreader), dried at 110°, and stored at room temperature exposed to the air for at least 18 h before use. Porphyrin methyl esters (up to 100 μg, depending on the composition of the mixture) in chloroform were applied as a compact spot at one corner of the plate, 3 cm from the margins. Development was carried out in the dark in tanks, previously saturated with solvent vapour, using the following solvent systems: (A) Carbon tetrachloride–dichloromethane–methyl acetate–methyl propionate (2:2:1:1)[9]. (B) Benzene–butanone (40:3). (C) Chloroform–kerosene–methanol (200:100:7), modified from the system of CHU AND CHU[2].

Development was carried out in system A until the solvent reached the top of the plate, which was then removed from the tank and dried in a stream of warm air. The plate was then turned through 90° developed similarly with system B and dried as before. The final development with system C was in the same direction as for system B, the solvent again being allowed to run to the top of the plate. After drying, the chromatogram was examined in visible and UV light (Wood's filter) to reveal porphyrins.

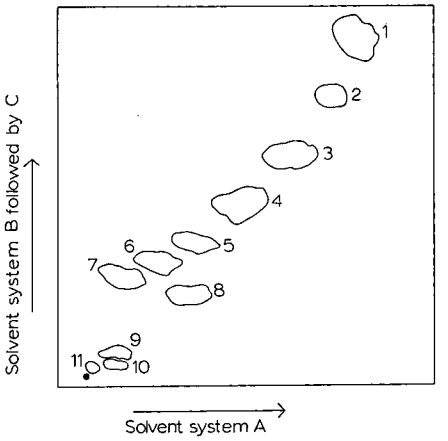

Fig. 1. Separation of a mixture of porphyrin methyl esters by two-dimensional TLC. 1 = mesoporphyrin IX, 2 = harderoporphyrin, 3 = coproporphyrin, 4,5,6 = penta-, hexa- and heptacarboxylic porphyrins, respectively, 7 = uroporphyrin, 8 = mono-β-hydroxypropionic monopropionic deuteroporphyrin IX, 9 = bis-β-hydroxypropionic deuteroporphyrin IX, 10 = haematoporphyrin IX, 11 = isohaematoporphyrin IX (all as their methyl esters).

Porphyrin methyl esters were eluted from the plates as described by CARDINAL *et al.*[4], and estimated by spectrophotometry, using correction factors[10].

Results and discussion

The separation of a mixture of porphyrin methyl esters is shown in Fig. 1. Improved resolution of the higher R_F porphyrins is obtained if development with system B is repeated, and of hydroxylated porphyrins by increasing the volume of methanol in system C (chloroform–kerosene–methanol, 200:100:15). Neither isomers of the I and III series, nor mixtures of protoporphyrin and mesoporphyrin IX dimethyl esters are separated. Porphyrin methyl esters with free carboxyl groups do not remain at the origin, but confusion with other low R_F porphyrin methyl esters can be prevented by comparing chromatograms obtained before and after re-esterification and acetylation[11] of the sample.

Although separations are reasonably reproducible, there is sufficient variation between chromatograms to make standardisation by internal markers desirable. Recoveries of porphyrin methyl esters are given in Table I. Destruction of proto-

TABLE I

RECOVERIES OF PORPHYRIN METHYL ESTERS AFTER TWO DIMENSIONAL TLC

	No. of estimations	*Range (%)*	*Mean (%)*
Coproporphyrin III tetramethyl ester	8	78–87	80
Mesoporphyrin IX dimethyl ester	4	88–96	92
Protoporphyrin IX dimethyl ester	4	32–59	49

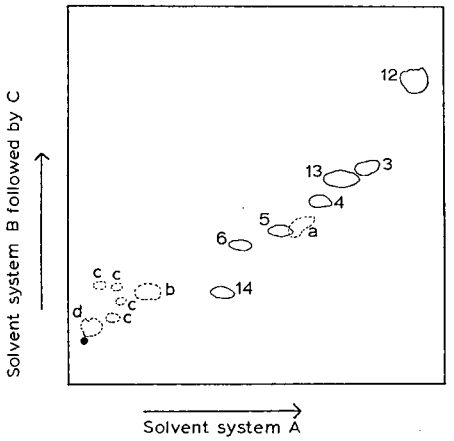

Fig. 2. Separation by two-dimensional TLC of the major porphyrins (continuous lines) and other pigments (dotted lines) extracted from the faeces of a patient with symptomatic porphyria. Pigments were esterified during extraction. 12 = dicarboxylic porphyrin, 13 = an unidentified tetracarboxylic porphyrin[15], 14 = "hydroxycoproporphyrin"[15], a = verdin, b = major violin, c = minor violins, d = brown pigment. Other porphyrins, see legend to Fig. 1.

porphyrin dimethyl ester on TLC in a solvent system containing benzene has been reported previously[3], and may partly account for the low recovery found.

This method has been used extensively to analyse faecal porphyrin excretion patterns (Fig. 2), and should be useful in the investigation of other material, for example bile[12], and normal and neoplastic liver tissue[13,14], where porphyrins with OH groups may otherwise be obscured by non-porphyrin pigments.

Thanks are due to Professor A. H. JACKSON for providing the sample of synthetic harderoporphyrin, and Professor C. H. GRAY for advice and encouragement.

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1 E. DEMOLE, *J. Chromatogr.*, 1 (1958) 24.
2 T. C. CHU AND E.J.-H. CHU, *J. Chromatogr.*, 21 (1966) 46.
3 M. DOSS, *J. Chromatogr.*, 30 (1967) 265.
4 R. A. CARDINAL, I. BOSSENMAIER, Z. J. PETRYKA, L. JOHNSON AND C. J. WATSON, *J. Chromatogr.*, 38 (1968) 100.
5 T. C. CHU, A. N. GREEN AND E. J.-H. CHU, *J. Biol. Chem.*, 190 (1951) 6343.
6 L. BOGORAD AND S. GRANICK, *J. Biol. Chem.*, 202 (1953) 793.
7 J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964, p. 176.
8 S. SANO, *J. Biol. Chem.*, 241 (1966) 5276.
9 M. S. STOLL AND C. H. GRAY, *Biochem. J.*, 117 (1970) 271.
10 J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964, p. 171.
11 J. BARRETT, *Nature*, 183 (1959) 1185.
12 S. G. SMITH, R. V. BELCHER, R. MAHLER AND J. YUDKIN, *Clin. Chim. Acta*, 23 (1969) 241.
13 S. G. SMITH, R. V. BELCHER AND R. MAHLER, *Biochem. J.*, 118 (1970) 38 P.
14 R. V. BELCHER, S. G. SMITH, D. C. NICHOLSON AND R. WILLIAMS, *Biochem. J.*, 119 (1970) 16P.
15 G. H. ELDER, *S. Afr. J. Lab. Clin. Med.*, (1971) in press.

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CHROM. 5349

Ion-exchange chromatography of calcium and magnesium in mixed dimethyl sulphoxide–water media

The effect of dimethyl sulphoxide (DMSO) on the anion-[1,2] and cation-exchange[3–6] behaviour of several elements has recently been investigated by several authors. JANAUER studied the ion-exchange properties of Ca^{2+}, Mg^{2+} and several other metal ions[4]. The present article describes in more detail the ion-exchange and the separation possibilities of Mg^{2+} and Ca^{2+} in this medium.

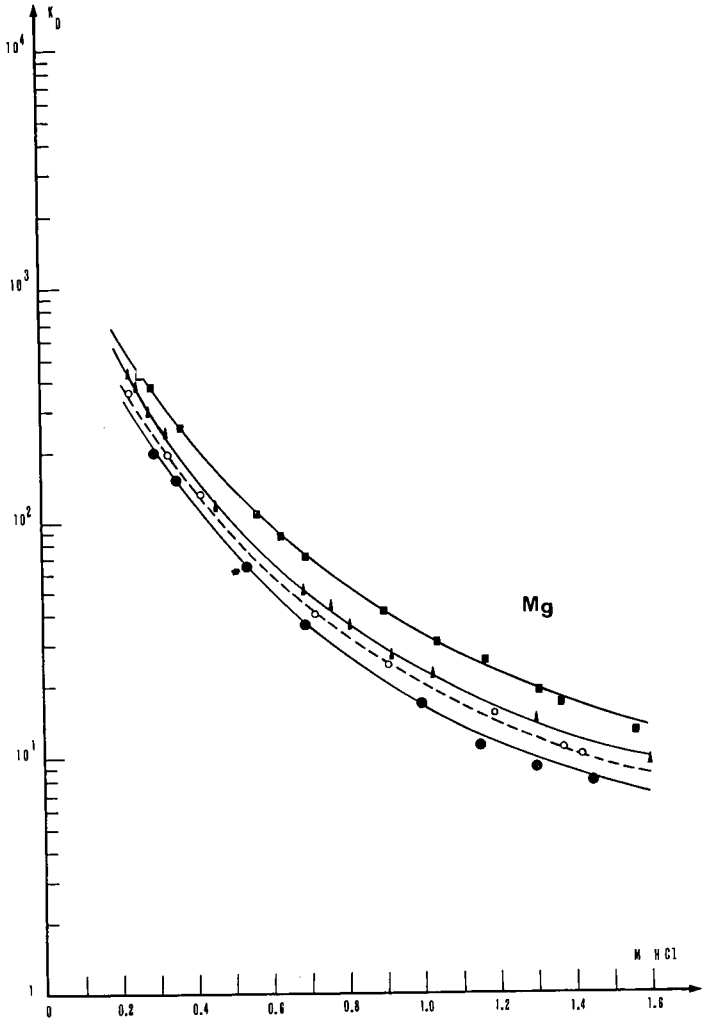

Fig. 1. Distribution coefficient of Mg^{2+} as a function of HCl concentration in mixtures of 0% (○— — —○), 35% (●—●), 50% (▲—▲) and 69% v/v DMSO (■—■).

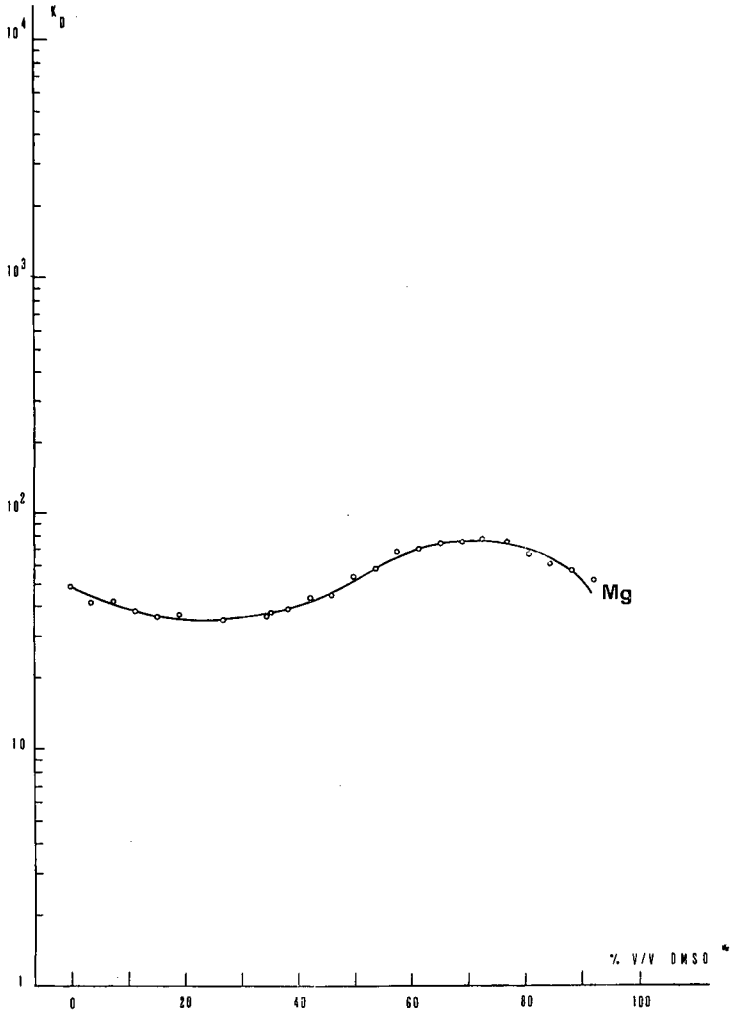

Fig. 2. Distribution coefficient of Mg^{2+} in mixtures of water –DMSO–HCl (overall concentration of HCl 0.69 *N*) as a function of the DMSO concentration.

Experimental

Apparatus. A Vitatron UC200 colorimeter microtitrator and a Radiometer automatic photometric titrator were used for the chelatometric determination of the metal ions.

Reagents. The DMSO used was spectrograde; all other reagents were reagent grade. Dowex 50W-X8 resin, 200–400 mesh, hydrogen-form, was used throughout this study. Chloride salts of Ca and Mg were used to prepare stock solutions containing 6 mg Ca/ml and 4 mg Mg/ml, respectively.

Procedure. The adsorbabilities, expressed as weight distribution coefficients, K_D, were determined by batch and column methods.

In batch experiments, exactly weighed 1.0000-g amounts of the dry, pre-

treated resin were equilibrated with 1 ml of the appropriate metal stock solution (containing 0.33 mequiv./ml of the metal ion) and 25 ml of a solvent mixture consisting of 0–100% of DMSO and 100–0% of hydrochloric acid, 1–12 *M*.

In all the column elution experiments, a column of 20 × 0.7 cm was used, packed with 1.0000 g of the dry pretreated resin. 500 μl of a mixture containing 2 mg Mg and 4 mg Ca were pipetted on the column, which was eluted at a flow rate of 0.2–0.3 ml/min.

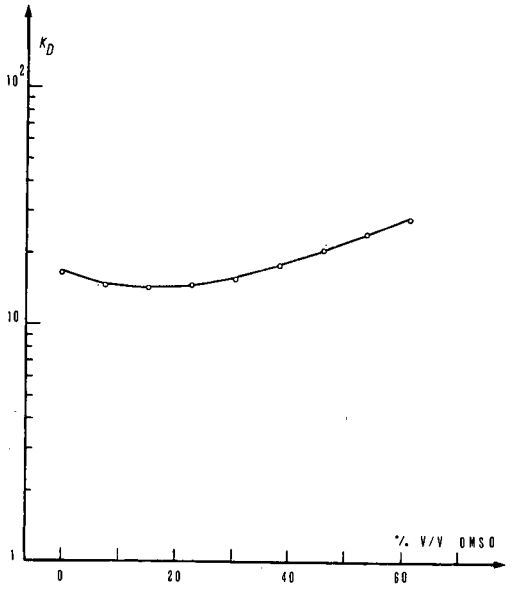

Fig. 3. Distribution coefficient of Mg^{2+} in mixtures of water–DMSO–HNO_3 (overall concentration of HNO_3 1.11 *N*) as a function of the DMSO concentration.

Results and discussion

The distribution constants decrease with increasing HCl molarities in all the DMSO–H_2O mixtures (Fig. 1). The influence of DMSO content on the distribution constants is shown in Fig. 2. A maximum is found at ± 70% v/v DMSO. At this composition (DMSO–H_2O molar ratio 1:2) a particularly stable structure of the liquid phase exist[7]. As noted by JANAUER[6], extreme values of viscosity, enthalpy of mixing, density, etc., occur at the same composition of the solution. According to the competitive solvation theory of ion-exchange selectivity[8], the maximum in K_D can be interpreted as an exclusion from a highly structured external phase of the ion which is least in need of solvation.

A more unexpected result is that a minimum is also found (at ± 30% v/v DMSO). Because it is known that anions are very reactive in DMSO media[7], it was thought that complexation of Mg^{2+} with Cl^- could be responsible for this phenomenon. However, exactly the same behaviour was found in DMSO–H_2O–HNO_3 mixtures (Fig. 3). Since NO_3^- is a much less reactive ligand than Cl^-, this makes the complexation hypothesis improbable.

Table I gives a number of K_D values for Ca^{2+} and Mg^{2+} in different media,

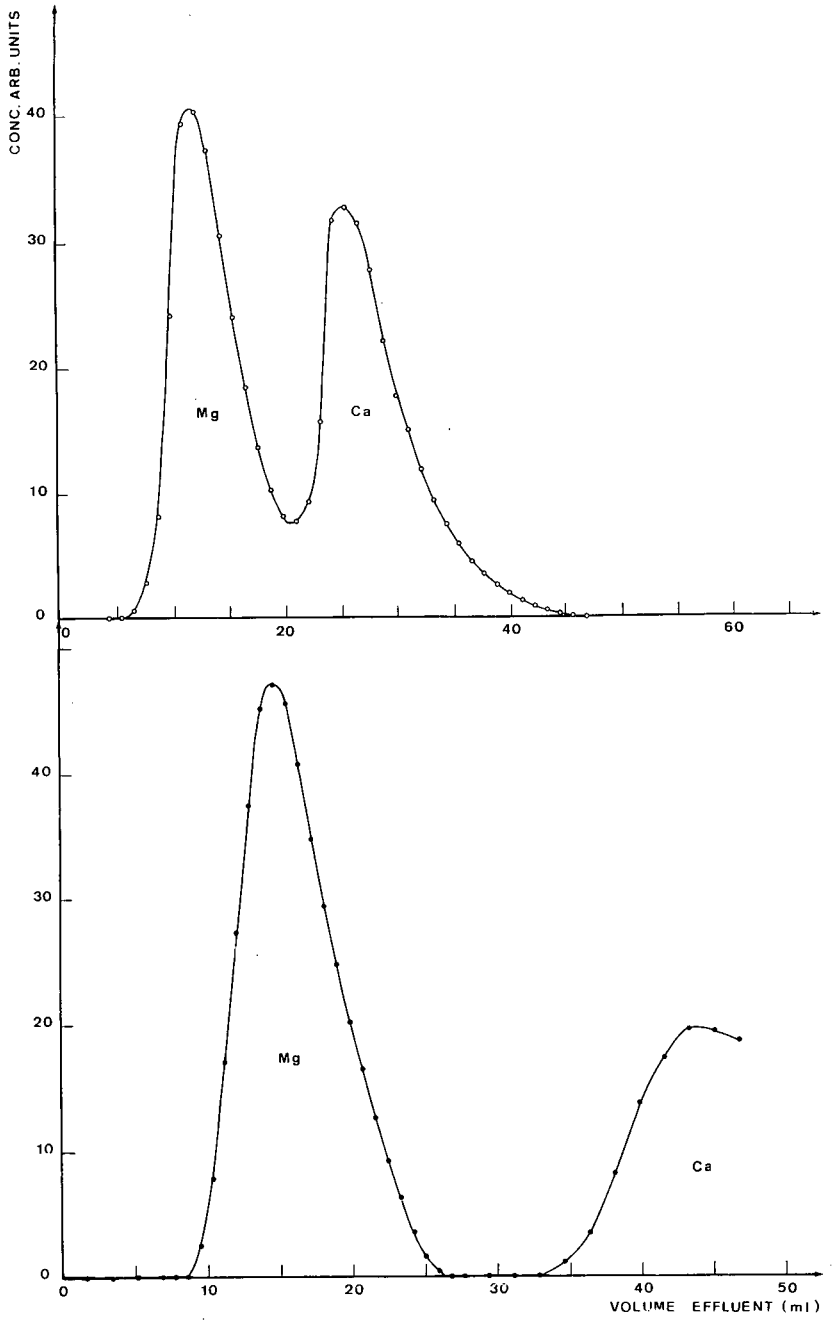

Fig. 4. Separation of Ca^{2+} and Mg^{2+} with 2.02 *N* HCl (○—○) and with 50% v/v DMSO/2.02 *N* HCl (●—●).

TABLE I

DISTRIBUTION AND SEPARATION COEFFICIENTS OF Mg^{2+} AND Ca^{2+}

DMSO (% v/v)	*HCl (N)*	K_D		β
		Mg^{2+}	Ca^{2+}	
0	1.01	18.8	47.4	2.5
0	2.02	5.12	11.8	2.3
35	2.61	2.41	6.90	2.9
35	3.37	1.68	5.20	3.1
35	4.67	0.61	4.04	6.6
50	1.24	13.3	43.0	3.2
50	2.02	7.27	21.4	2.9
50	2.37	3.53	15.3	4.3
50	3.08	2.36	9.76	4.1
50	4.50	2.05	7.33	3.6

and the corresponding separation factors, β. The β-value is larger in DMSO media, so that better separations should be possible. This was tested with several media of different composition. It was found that good separations can be achieved with quite small volumes of eluting agents. However, the separations are no better or only slightly better than separations in pure aqueous media (Fig. 4). This is due to the fact that the increase in β is offset by an increase of the HETP. This is not surprising since in the range considered, the viscosities increase with increasing DMSO content[9]. Therefore, the diffusion coefficient must decrease. Taking into account Glueckauf's equation for the HETP, this must lead to an increase in HETP. Because of this phenomenon, the addition of organic solvents does not always result in better resolution. In order to be able to evaluate the effect of solvent composition on HETP, a systematic investigation of HETP as a function of the composition of the mixed aqueous–organic eluting solution is now being carried out at this laboratory.

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1 A. M. PHIPPS, *Anal. Chem.*, 40 (1968) 1769.
2 J. S. FRITZ AND M. L. GILETTE, *Talanta*, 15 (1968) 287.
3 I. BIRZE, L. W. MARPLE AND H. DIEHL, *Talanta*, 15 (1968) 1441.
4 G. E. JANAUER, *Mikrochim. Acta*, 6 (1968) 1111.
5 E. BLASIUS AND R. SCHMITT, *J. Chromatogr.*, 42 (1969) 53.
6 G. E. JANAUER, H. E. VAN WART AND J. T. CARRANO, *Anal. Chem.*, 42 (1970) 215.
7 A. J. PARKER, *Quart. Rev.*, 16 (1962) 163.
8 R. M. DIAMOND AND D. C. WHITNEY, in J. A. MARINSKY (Editor), *Ion Exchange*, Vol. 1, Marcel Dekker, New York, 1966, p. 286.
9 G. EBERT AND J. WENDORFF, *Ber. Bunsenges. Physik. Chem.*, 74 (1970) 1071.

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Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

ANGELE, H. P. (Editor): *Four-Language Technical Dictionary of Chromatography*, Pergamon, Oxford, 1970, 120 pp.—PC and TLC.

HAIS, I. M.: Isolation and purification procedures. I. Chromatography. In Z. M. BACQ (Editor): *Fundamentals of Biochemical Pharmacology*, Pergamon, Oxford, 1970, pp. 35–44—PC and TLC; a review.

OGUMA, K., ISHIDA, K., KURODA, R., EGASHIRA, S., MIYAZAKI, M. AND HATANO, H.: Chromatographic analysis. *Japan Analyst*, annual review (1970) 74R–92R—703 references; PC and TLC.

SAVIDAN, L.: *La chromatographie (Monographies Dunod, 5)*, Dunod, Paris, 1970, 130 pp.; *C.A.*, 72 (1970) 115162h.

2. FUNDAMENTALS, THEORY AND GENERAL

KWAPNIEWSKI, Z., SLIWIOK, J., JOHN, A., MORAWSKI, T., WEGRZYNEK, J. AND RZEPA, J.: (Melting point, paper chromatography and thin-layer chromatography as criteria of organic compound purity). *Zesz. Nauk. Politech. Krakow., Chem.*, No. 1 (1968) 33–40; *C.A.*, 72 (1970) 139424w—PC and TLC.

RHEE, H.: Theory of chromatography. *Avail. Univ. Microfilms, Ann Arbor, Mich.*, Order No. 69-11,503; *C.A.*, 72 (1970) 125370p—chromatography in general.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

GILTROW, J.: (Continuous separation of mixture components). *Ger. Pat., Offen.* 1,951,064 (Cl. G 01*n*) 16 Apr 1970; Brit. Appl. 15 Oct 1968–25 Feb 1969, 19 pp.; *C.A.*, 72 (1970) 139406s.

KWAPNIEWSKI, Z., SLIWIOK, J., RZEPA, J., OGIERMAN, L. AND HOFFMANN, B.: (Characteristics of selected chromatographic papers). *Zesz. Nauk. Politech. Krakow., Chem.*, No. 4 (1968) 13–22; *C.A.*, 72 (1970) 10329k.

SHUKLA, P. K. AND DUBEY, S. S.: A semiautomatic sprayer for paper chromatography. *Sci. Tools*, 17 (1970) 33–34.

7. PHENOLS

MACDONALD, B. F. AND SWAN, E. P.: Chromatography of the lignans of *Thuja plicata* Donn. *J. Chromatogr.*, 51 (1970) 553–555—PC and TLC.

TAIMR, L. AND POSPÍŠIL, J.: The use of R_C values for structural studies in the paper chromatography of phenols. *J. Chromatogr.*, 52 (1970) 131–137—R_C values for 86 compounds.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

HUBÁCEK, J.: Paper chromatography of flavonoids from hops (*Humulus lupulus* L.). III. Identification of apigenin, apigenin-7-glucoside and quercetin-7-glucoside. *Coll. Czech. Chem. Commun.*, 35 (1970) 3119–3123—PC and TLC on polyamide layers.

9. OXO COMPOUNDS

SIMATUPANG, M. H. AND HAUSEN, B. M.: Chromatographische Nachweisreaktionen von chinoiden Verbindungen. *J. Chromatogr.*, 52 (1970) 180–183—PC and TLC.

10. CARBOHYDRATES

10a. Mono and oligosaccharides: structural studies

ASPINALL, G. O. AND SANDERSON, G. R.: Plant gums of the genus Sterculia. IV. Acidic oligosaccharides from *Sterculia ureus* gum. *J. Chem. Soc. (C)*, (1970) 2256–2258.

ASPINALL, G. O. AND SANDERSON, G. R.: Plant gums of the genus Sterculia. V. Degradation of carboxy-reduced *Sterculia ureus* gum. *J. Chem. Soc. (C)*, (1970) 2259–2264.

STROBEL, G. A.: A phytotoxic glycopeptide from potato plants infected with *Corynebacterium sepedonicum*. *J. Biol. Chem.*, 245 (1970) 32–38—R_F values for 9 sugar acids.

VENIAMIN, M. P.: Computer-assisted microanalysis of complex saccharides. I. Studies on the re-

peating sequence of the *Klebsiella aerogenes* NCTC 9644 capsular polysaccharide. *J. Chromatogr. Sci.*, 8 (1970) 173–176.

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

ALIEV, A. M. AND ALEKPEROV, A. F.: (Stage-wise control of heparin production using paper chromatography). *Khim. Farm. Zh.*, 4, No. 2 (1970) 48–50; *C.A.*, 72 (1970) 136337j.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

KWAPNIEWSKI, Z., SLIWOK, J., AND BUHL, F.: (Paper chromatography in the determination of fatty acid purity). *Zesz. Nauk. Politech. Krakow., Chem.*, No. 1 (1968) 17–23; *C.A.*, 72 (1970) 139480m.

KWAPNIEWSKI, Z., SLIWIOK, J., BUDNIOK, A. AND WIELGOS, E.: (Effect of the interfacial tension of the moving phase on the R_F values of fatty acids in partition chromatography). *Zesz. Nauk. Politech. Krakow., Chem.*, No. 4 (1968) 23–29; *C.A.*, 72 (1970) 136766y.

VERKHOVTSEVA, T. P., LUR'E, L. M. AND LEVITOV, M. M.: (Chromatographic method for determining phenylacetic acid in a culture medium of *Penicillium chrysogenum*). *Khim. Farm. Zh.*, 3, No. 12 (1969) 52–54; *C.A.*, 72 (1970) 120036z.

See also TLC section.

11b. Lipids and their constituents

See TLC section.

13. STEROIDS

BALL, P. AND BREUER, H.: Anreichung und Charakterisierung einer cytoplasmatischen 17-Hydroxysteroid: NAD(P)-Oxidoreduktase aus der Kaninchenleber. *Z. Physiol. Chem.*, 351 (1970) 1011–1025—PC and TLC.

See also TLC section.

14. STEROID GLYCOSIDES AND SAPONINS

TSARENKO, N. YA. AND SHRAIBER, M. S.: (Spectrophotometric method for the determination of gomphotin in *Gomphocarpus fruticosus*) 13, *Farmatsiya (Moscow)*, 19, No. 2 (1970) 39–41; *C.A.*, 73 (1970) 7297t.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

KALLISTRATOS, G., PFAU, A. AND TIMMERMANN, A.: Untersuchungen über Malignolipin. *Z. Krebsforsch.*, 73 (1970) 387–396.

KASEDA, H., NOGUCHI, T., KIDO, R. AND MATSUMURA, Y.: The isolation and identification of 2-amino-3-hydroxyacetophenone from the urine of rats. *Experientia*, 26 (1970) 828–829—PC and TLC.

NAKAJIMA, T., ZACK, JR., J. F. AND WOLFGRAM, F.: *N*-Monoacetylspermidine as a normal constituent of urine. *Biochim. Biophys. Acta*, 184 (1969) 651–652.

18. AMINO ACIDS

COLMAN, R. F. AND CHU, R.: The role of sulfhydryl groups in the catalytic function of isocitrate dehydrogenase. III. Effect of N-ethylmaleimide on chemical and physical properties. *J. Biol. Chem.*, 245 (1970) 608–615.

MATSUOKA, Y., KUMON, A., NAKAJIMA, T., KAKIMOTO, Y. AND SANO, I.: Identification of ε-N-methyl-L-lysine and δ-N-methylornithine in bovine brain. *Biochim. Biophys. Acta*, 192 (1969) 136–137.

SMITH, R. C. AND POLLARD, D. R.: Excretion of *S*-adenosylthionine in the urine of rats fed a diet containing ethionine. *Biochim. Biophys. Acta.*, 184 (1969) 397–403.

WITTMANN, H., MÜLLER, A. K. AND ZIEGLER, E.: Neue Reagentien zur papierchromatographischen Nachweis von Aminosäuren. 6. Zur Kenntnis der "Ninhydrin-reaktion". *Monatsh. Chem.*, 101 (1970) 1388–1393.

See also TLC section.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

CHIOSA, L., BOTTESCH, A. AND BONCIOCAT, N.: (Determination of pharmaceutical ACTH products

(adrenocortical hormone) by paper chromatography). *Farmacia (Bucharest)*, 17 (1969) 713–720; *C.A.*, 72 (1970) 136473a.

LAPIDOT, Y., DE GROOT, N. AND RAPPOPORT, S.: Peptidyl transfer RNA. VI. The chemical synthesis of tri- and tetrapeptidyl transfer RNA. *Biochim. Biophys. Acta.*, 182 (1969) 105–112.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

CARPENTER, J. M. AND SHAW, G.: Purines, pyrimidines and imidazoles. Part XXXIII. Synthesis of 5-methylsulphonyluridine and its 5'-mono- and pyro-phosphates. *J. Chem. Soc. (C)*, (1970) 2016–2022—R_F values for 13 compounds.

CHIRIKDJIAN, J. G. AND DAVIS, F. F.: Compositional variations in the common pentanucleotide from transfer ribonucleic acids of *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1296–1301—PC and TLC.

HACKER, B.: Enzymatic phosphorylation of *N*-(3-methylbut-2-enyl)adenosine by L1210 leukemic cells. *Biochim. Biophys. Acta*, 224 (1970) 635–638.

TUMAITIS, T. D. AND LANE, B. G.: Differential labelling of the carboxymethyl and methyl substituents of 5-carboxymethyluridine methyl ester, a trace nucleoside constituent of yeast transfer RNA. *Biochim. Biophys. Acta*, 224 (1970) 391–403.

25. ORGANIC PHOSPHORUS COMPOUNDS

KHWAJA, T. A., REESE, C. B. AND STEWART, J. C. M.: A convenient general procedure for the conversion of alcohols into their monophosphate esters. *J. Chem. Soc. (C)*, (1970) 2092–2100.

27. VITAMINS AND VARIOUS GROWTH FACTORS

KAZARINOVA, G. I.: (Nessler reagent as a developer for ascorbic acid during detection by paper chromatography). *Uch. Zap., Mariiskii Gos. Pedagog. Inst.*, 30 (1968) 69–71; *C.A.*, 73 (1970) 7290k.

REMBOLD, H., METZGER, H., SUDERSHAN, P. AND GUTENSOHN, W.: Catabolism of pteridine cofactors. I. Properties and metabolism in rat liver homogenates of tetrahydrobiopterin and tetrahydroneopterin. *Biochim. Biophys. Acta*, 184 (1969) 386–396.

VALERINO, D. M. AND MCCORMACK, J. J.: Studies of the oxidation of some aminopteridines by xanthine oxidase. *Biochim. Biophys. Acta*, 184 (1969) 154–163—R_F values for 12 pteridines.

WATANABE, H.: Separation of B group vitamins using ion exchange paper. Part 2. *Japan Analyst*, 19 (1970) 1658–1663.

28. ANTIBIOTICS

See TLC section.

30. SYNTHETIC AND NATURAL DYES

KITAHARA, S.: Paper and thin-layer chromatography of dyes intermediates. *Senshoku Kogyo*, 17 (1970) 654–661; *C.A.*, 72 (1970) 134112h—a review; PC and TLC; R_F values for 165 compounds.

KRESSE, P.: (Chromatographic method for the determination of the flocculation tendency of pigments). *Farbe Lack*, 76 (1970) 351–358; *C.A.*, 72 (1970) 134219y—paper coated with silica gel.

MATRKA, M., CHMÁTAL, V., PÍPALOVÁ, J., SÁGNER, Z., KROUPA, J. AND MARHOLD, J.: Oxydation karzinogener Azofarbstoffe. V. Oxydation *meta*-substituierter Derivate des N,N-dimethyl-4-aminoazobenzols mit Hilfe von Cer (IV)-sulfat und einigen weiteren Oxydationsmitteln sowie auf elektrochemischen Weg. *Coll. Czech. Chem. Commun.*, 35 (1970) 2944–2953—many R_F values.

See also TLC section.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

CHODAKOWSKI, A. M.: (Chromatographic method of separation of phenothiazine derivatives and their degradation products). *Farm. Pol.*, 25 (1969) 637–641; *C.A.*, 73 (1970) 7109h—PC and TLC; a review with 39 references.

FIUSSELLO, N.: (Detection and determination of psilocybin). *Mycopathol. Mycol. Appl.*, 39 (1969) 329–335; *C.A.*, 72 (1970) 136482—PC and TLC.

PAWELCZYK, E. AND WACHOWIAK, R.: (Chemical characterization of the products of drug de-

composition. IV. Mechanism of phenylbutazone decomposition in pharmaceutical preparations). *Acta Pol. Pharm.*, 26 (1969) 433–438; *C.A.*, 72 (1970) 136355p.

RUGGIERI, R.: (Physical, chemical, chromatographic and electrophoretic properties of pralidoxime methiodide). *Boll. Chim. Farm.*, 108 (1969) 611–618; *C.A.*, 73 (1970) 7334c.

STEBLETSOVA, ZH. D.: (Stability of a soliutizon solution). *Khim. Farm. Zh.*, 4, No. 2 (1970) 43–46; *C.A.*, 72 (1970) 136453u.

See also TLC section.

32c. Plant extracts

MURTY, B. S. R., VASUDEVAN, T. N. AND KHORANA, M. L.: Estimation of cucurbitacins in medicinal plants. *Indian J. Pharm.*, 32 (1970) 10; *C.A.*, 72 (1970) 136486g.

33. INORGANIC SUBSTANCES

ADCOCK, L. H. AND HOPE, W. G.: A method for the determination of tin in the range 0.2 to 1.6 μg and its application to the determination of organotin stabiliser in certain foodstuff. *Analyst*, 95 (1970) 868–874.

ALESKOVSKAYA, V. N. AND ALESKOVSKII, V. B.: (Determination of micro-amounts of certain cations by peak precipitation chromatography on paper impregnated with slightly soluble diethyl dithiocarbamates). *Zh. Anal. Khim.*, 25 (1970) 243–249; *C.A.*, 73 (1970) 10423m.

MARCU, G. AND TOMUS, M.: (Reaction of sodium and ammonium molybdate solutions with nitrilotriacetic acid. I. Chromatographic and electroradiochromatographic study). *Stud. Univ. Babes-Bolyai, Ser. Chem.*, 14, No. 2 (1969) 23–26; *C.A.*, 73 (1970) 10255h.

MICHAL, J.: (*Chromatography in Inorganic Analysis*) SNTL, Prague, 1970, 258 p.—mostly PC, but also TLC and other chromatography techniques.

THAKUR, M. L.: The separation of some elements with the use of tri-*n*-butyl orthophosphate. *Separ. Sci.*, 5 (1970) 645–651.

VAN RAAPHORST, J. G. AND HAREMAKER, H. G.: Separation of sodium and potassium by ion exchange on cellulose phosphate. *Talanta*, 17 (1970) 345–349; *C.A.*, 73 (1970) 10340g.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

ONICIU, L.: Improvement in the radiochemical purity determination of sodium radiochromate (^{51}Cr) by paper chromatography. *Radiochem. Radioanal. Lett.*, 3 (1970) 103–112; *C.A.*, 72 (1970) 128434y.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

JÄNCHEN, D. (Editor): *Thin-layer Chromatography. Cumulative Bibliography II (1967–1969).* Camag, Muttenz, 1970—about 3000 references, however no index of compounds and authors.

RAYBURN, J. A., ARNOLD, L. B., HOFFMAN, J., LAWSON, W. H., NETTLES, J. E., SMITH, A. L. AND WHITE, E.: Thin-layer chromatography, an overlooked analytical tool. *Text. Chem. Color.*, 1, No. 2 (1969) 58–66; *C.A.*, 72 (1970) 122681e—a review; applications to textile problems.

STAHL, E. (Editor): *Chromatographische und mikroskopische Analyse von Drogen.* G. Fischer, Stuttgart, 1970, 195 pp.

See also PC section.

2. FUNDAMENTALS, THEORY AND GENERAL

See PC section.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

CHUDZIK, J. AND KLEIN, A.: (Thin-layer Sephadex gel filtration). *Wiad. Chem.*, 23 (1969) 847–863; *C.A.*, 72 (1970) 128463g—a review with 32 references.

CORDIS CORP.: Applicator for chromatography and electrophoresis. *Brit. Pat.*, 1, 177, 730 (Cl. B 01*d*), 14 Jan 1970; U.S. Appl. 19 Jun 1967; *C.A.*, 72 (1970) 123325d.

DeThomas, A. V. and Zdankiewicz, M.: Simple pre-equilibration apparatus for thin-layer chromatography. *Microchem. J.*, 15 (1970) 74–77; *C.A.*, 72 (1970) 139243m.
DeThomas, A. V., Zdankiewicz, M. and Witkovic, D.: Modified "sandwich" microapparatus for descending thin-layer chromatography. *Microchem. J.*, 15 (1970) 71–73; *C.A.*, 72 (1970) 139233h.
Grace, W. R., and Co.: Plates for thin-layer chromatography. *Brit. Pat.*, 1, 181,089 (Cl. B 01*d*), 11 Feb 1970; U.S. Appl. 21 Dec 1966; 2 pp.; *C.A.*, 72 (1970) 139487u—glass plates coated with TiO_2.
Gregoriwicz, Z. and Sliwiok, J.: Indexes for estimation of developing reagents in thin-layer chromatography. *Microchem. J.*, 15 (1970) 60–63; *C.A.*, 72 (1970) 139224f.
Heinisch, E. and Hornawsky, G.: Zur quantitativen Auswertung von Dünnschichtchromatogrammen mit dem ERI 65 aus Jena. *Jenaer Rundsch.*, 15 (1970) 130–135.
Jaworek, D.: (Thin-layer chromatography apparatus). *U.S. Pat.*, 3,501,009 (Cl. 210–198; B 01*d*), 17 Mar 1970; Ger. Appl. 21 Nov 1968, 5 pp.; *C.A.*, 72 (1970) 125409h.
Nicolaides, N.: Magnesium oxide as an adsorbent for the chromatographic separation of molecules according to their degree of flatness, *e.g.* the separation of wax esters from sterol esters. *J. Chromatogr. Sci.*, 8 (1970) 717–720.
Sandroni, S. and Schlitt, H.: An arrangement for simultaneous elution of one thin-layer chromatography plate with several solvents. *J. Chromatogr.*, 52 (1970) 169–171—modified KS chamber with longitudinal barriers.
Saunders, D. L. and Snyder, L. R.: Drum TLC. A new technique for rapid, high efficiency separations. *J. Chromatogr. Sci.*, 8 (1970) 706–716.
Simonides, J., Nádvorník, V. and Pliml, J.: (Support for ascending or descending thin-layer chromatography). *Czech. Pat.*, 131,935 (Cl. G 01*n*), 15 Apr 1969, Appl. 31 Jan 1967; 2 pp.; *C.A.*, 73 (1970) 10458b—poly(ethylene terephthalate) etc. are bonded with Al sheet.
Slining, K. D.: Cellulose-coated chromatographic sheets. *U.S. Pat.*, 3,498,830 (Cl. 117–138.8; C 08*dj*, B 01*d*), 03 Mar 1970, Appl. 01 Mar 1967, 2 pp.; *C.A.*, 72 (1970) 123198q.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

Collins, R. F.: Thin-layer chromatography plates for samples the major portion of which migrates very little during development. *Brit. Pat.*, 1,183,836 (Cl. B 01*d*), 11 Mar 1970; Appl. 04 Sep 1967; 5 pp.; *C.A.*, 72 (1970) 128449g.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

Matthews, P. J.: Thin-layer chromatography for identification of petroleum and coal-tar oils and residues. *J. Appl. Chem.*, 20, No. 3 (1970) 87–92; *C.A.*, 72 (1970) 113454f.
Youngs, V. L. and Gilles, K. A.: Wheat hydrocarbons. *Cereal Chem.*, 47 (1970) 317–323.

6. ALCOHOLS

Kwapniewski, Z., Sablik, J., Przybyla, H. and Nizinska, M.: (Thin-layer chromatography and ionophoresis in the determination of the purity of phytin compounds). *Zesz. Nauk. Politech. Krakow., Chem.*, No. 1 (1968) 25–31; *C.A.*, 72 (1970) 136490d.

7. PHENOLS

Barni, I. and Casini, A.: (Determination of apiol by chromatography on thin layers). *Zucchia*, 4 (1968) 329–334; *C.A.*, 72 (1970) 119578w.
Kutlu, H.: Determination of impurities in resorcinol by thin-layer chromatography. *Istanbul Univ. Eczacilik Fak. Mecm.*, 5 (1969) 98–102; *C.A.*, 72 (1970) 136481b.
See also PC section.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

Chambon, P. and Chambon-Mougenot, R.: Contribution á l'identification des résines toxiques du Chanvre. *Ann. Pharm. Fr.*, 27 (1969) 739–742.
Dutton, M. F. and Heathcote, J. G.: O-Alkyl derivatives of aflatoxins B_{2a} and G_{2a}. *Chem. Ind. (London)*, (1969) 983–986.
Grlić, L.: A highly sensitive chromatographic detection of Cannabis constituents by means of silica gel sheets treated with silver nitrate. *Acta Pharm. Jugoslav.*, 20 (1970) 19–22.
Sasaki, M., Kaneko, Y., Oshita, K., Takamatsu, H. and Yokotsuka, T.: Studies on the compounds produced by molds. VII. Isolation of isocoumarin compounds. *Agr. Biol. Chem. (Tokyo)*, 34 (1970) 1296–1300.
Stubblefield, R. D., Shotwell, O. L., Shannon, G. M., Weisleder, D. and Rohwedder,

W. K.: Parasiticol: A new metabolite from *Aspergillus parasiticus*. *J. Agr. Food Chem.*, 18 (1970) 391–393.
See also PC section.

9. OXO COMPOUNDS

HARADA, K., MIURA, S., SHINODA, Y. AND YAMADA, K.: Thin-layer chromatographic identification of formaldehyde in aquatic animals. *Nippon Suisan Gakkaishi*, 36 (1970) 188–191; *C.A.*, 72 (1970) 118313u.
See also PC section.

10. CARBOHYDRATES

10a. Mono and oligosaccharides: structural studies

HANGYAL, K. AND WOLLER, L.: (Determination of raffinose in molasses). *Cukoripar*, 23, No. 1 (1970) 10–14; *C.A.*, 73 (1970) 5184e.

LATO, M., BRUNELLI, B., MEZZETTI, T., LEONE, P. AND CIUFFINI, G.: (Separation of carbohydrates by thin-layer chromatography on silica gel). *Quad. Nutr.*, 28, No. 3–4 (1968) 89–108; *C.A.*, 72 (1970) 129263d.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

ABATE, V., BADOUX, V., HICKS, Z. S. AND MESSINGER, M.: Characterization of natural and synthetic waxes (in cosmetics) using combined chromatographic techniques. *J. Soc. Cosmet. Chem.*, 21 (1970) 119–128; *C.A.*, 72 (1970) 136289v.

GRYNBERG, H. AND CEGLOWSKA, K.: (Study of the composition of vegetable oil glycerides by thin-layer chromatography on silica impregnated with silver nitrate. I. Determination of the relative complexing strength of fatty acid esters with silver ions). *Rev. Fr. Corps Gras*, 17, No. 2 (1970) 89–91; *C.A.*, 72 (1970) 134425n.

HOTELLIER, F. AND DELAVEAU, P.: Localisations histochimiques. I. Observations sur la diffusion de polyphénols chez les plantes vivantes. *Ann. Pharm. Fr.*, 28 (1970) 401–408—TLC and PC.

KATING, H., RINN, W. AND WILLUHN, G.: (Components of Arnica species. III. Fatty acids in the essential oils of Arnica blossoms of different varieties). *Planta Med.*, 18 (1970) 130–146; *C.A.*, 72 (1970) 124982c.

PACE-ASCIAK, C., MORAWSKA, K. AND WOLFE, L. S.: Metabolism of prostaglandin $F_{1\alpha}$ by the rat stomach. *Biochim. Biophys. Acta*, 218 (1970) 288–295.

PACE-ASCIAK, C. AND WOLFE, L. S.: Biosynthesis of prostaglandins E_2 and $F_{2\alpha}$ from tritium-labelled arachidonic acid by rat stomach homogenates. *Biochim. Biophys. Acta*, 218 (1970) 539–542.

STOLL, U.: Die Kombination von Dünnschicht- und Gaschromatographie bei der Identifizierung aliphatischer Hydroxy- und Dicarbonsäuren. *J. Chromatogr.*, 52 (1970) 145–151.

TOMEO LACRUE, M. AND ORO, M. A.: (Chromatographic analysis of corn stalk extracts). *Rev. Acad. Cienc. Exactas, Fis.-Quim. Natur. Zaragoza*, 23 (1968) 203–206; *C.A.*, 72 (1970) 118315w.

11b. Lipids and their constituents

BOLLINGER, J. N. AND SEELIG, L. L.: Adherence of vegetable bath oils to keratin. *J. Soc. Cosmet. Chem.*, 21 (1970) 613–623.

BRADY, R. O. AND MORA, P. T.: Alteration in ganglioside pattern and synthesis in SV_{40}- and polyoma virus-transformed mouse cell lines. *Biochim. Biophys. Acta*, 218 (1970) 308–319.

BRAUN, P. E., MORELL, P. AND RADIN, N. S.: Synthesis of C_{18}- and C_{20}-dihydrosphingosines, ketodihydrosyhingosines, and ceramides by microsomal preparations from mouse brain. *J. Biol. Chem.*, 245 (1970) 335–341.

CULP, T. W., TUCKER, P. W., RATLIFF, C. R. AND HALL, F. F.: Chromatographic analysis of ocular lipids. I. Bovine and human iris tissue. *Biochim. Biophys. Acta*, 218 (1970) 259–268.

GETZ, G. S., JAKOVCIC, S., HEYWOOD, J., FRANK, J. AND RABINOWITZ, M.: A two-dimensional thin-layer chromatographic system for phospholipid separation. The analysis of yeast phospholipids. *Biochim. Biophys. Acta*, 218 (1970) 441–452.

GORDON, S. G., PHILIPPON, F., BORGEN, K. S. AND KERN, JR., F.: Formation of fatty acid ethyl esters during lipid extraction and storage: an important artifact. *Biochim. Biophys. Acta*, 218 (1970) 366–368.

KRAMER, J. K. G. AND MANGOLD, H. K.: Preparation and characterisation of neutral diol plasmalogens. *Chem. Phys. Lipids*, 4 (1970) 332–344.

MORELL, P. AND RADIN, N. S.: Specificity in ceramide biosynthesis from long chain bases and various fatty acyl coenzyme A's by brain microsomes. *J. Biol. Chem.*, 245 (1970) 342–350—TLC and PC.

PEYPOUX, F. AND MICHEL, G.: Étude des phospholipides d'une souche sauvage et d'une souche mutante thermosensible d'*Escherichia coli*. *Biochim. Biophys. Acta*, 218 (1970) 453–462—TLC and PC.

PRUTHI, T. D. AND BHATIA, I. S.: Lipids in cereals. I. *Pennisetum typhoideum*. *J. Sci. Food Agr.*, 21 (1970) 419–422.

SLOMIANY, B. L. AND HOROWITZ, M. I.: The glycolipids of bovine serum. *Biochim. Biophys. Acta*, 218 (1970) 278–287.

SPENCER, F., MANGOLD, H. K., SANSONE, G. AND HAMILTON, J. G.: Long-chain alkyl acetates in the preputial gland of the mouse. *Biochim. Biophys. Acta*, 192 (1969) 516–521.

TAO, R. V. P. AND SWEELEY, CH. C.: Occurrence of hematoside in human plasma. *Biochim. Biophys. Acta*, 218 (1970) 372–375.

13. STEROIDS

DE SOUSA CORREIA, J. AND AFONSO, C. C.: (Mestranol + norethisterone. Analytic control of tablets). *Bol. Fac. Farm. Univ. Coimbra, Ed. Cient.*, 28 (1968) 53–59; *C.A.*, 72 (1970) 136474b.

FILLMANN, B. AND BREUER, H.: Qualitative und quantitative Untersuchung der mikrosomalen 7α(β)-Hydroxysteroid: NADP-Oxidoreduktase der Schweineleber. *Z. Physiol. Chem.*, 351 (1970) 1100–1112—TLC and PC.

JACOBS, J. AND MEIJER, J. W. A.: (Iodine vapor as a non-destructive localising agent in the thin-layer chromatography of steroids). *Tijdschr. Chem. Instrum.*, 13 (1969) 341–344; *C.A.*, 72 (1970) 118112c.

KSENOFONTOVA, E. V., MUKHINA, M. V. AND KHALETSKII, A. M.: (Determination of the composition of neutral products of β-sitosterol oxidation). *Farmatsiya (Moscow)*, 19, No. 1 (1970) 40–43; *C.A.*, 72 (1970) 136477e.

VOLKOVA, V. I., GAZIEV, G. A. AND POPOVA, L. A.: (Two-dimensional chromatography of a mixture of corticosteroids on a thin layer of KSK silica gel). *Vop. Med. Khim.*, 16, No. 1 (1970) 101–103; *C.A.*, 72 (1970) 129030a.

WENZEL, M. AND MÜTZEL, W.: Untersuchungen über den Wasserstofftransfer von Östradiol-17β auf 5α-Androsten-17-on im Rattengewebe. *Z. Physiol. Chem.*, 351 (1970) 1221–1231.

YEN, K.-Y., LEE, W.-H., CHEN, F.-H. AND LEE, T.-CH.: Analysis of medicinal products by thin-layer chromatography. II. Analysis and identification of steroidal hormones. *T'ai-Wan Yao Hsueh Tsa Chih*, 19, No. 12 (1967) 21–27; *C.A.*, 73 (1970) 7287q.

ZIVANOV STAKIC, D. AND MLADENOVIC, M.: (Thin-layer chromatographic identification of β-sitosterol in oil from *Helleborus odorus* roots and rhizomes). *Glas. Hem. Drus., Beograd*, 33 (1968) 311–315; *C.A.*, 73 (1970) 7288r.

See also PC section.

14. STEROID GLYCOSIDES AND SAPONINS

WAGNER, H., HÖRHAMMER, L. AND REBER, H.: Quantitative Bestimmung der Haupt und Nebenglykoside verschiedener Erysimum-Arten. *Arzneimittelforsch.*, 20 (1970) 215–218.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

IVESON, P. AND PARKE, D. V.: The preparation and chemical properties of some glucuronyl esters of β-glycyrrhetic acid (3β-hydroxy-11-oxo-olean-12-en-30-oic acid) and its derivatives. *J. Chem. Soc. (C)*, (1970) 2038–2042.

KARETNIKOVA, A. I., KARPOVA, T. I. AND KUSTOVA, S. D.: (Composition of carbonyl compounds from Soviet lavender oil). *Maslo-Zhir. Prom.*, 36, No. 2 (1970) 28–30; *C.A.*, 73 (1970) 7083v.

KRAUS, L., TRAN-THI-HOANG AND NGUYEN-THI-KIM CHI: (Quantitative determination of ascaridole in chenopodium oil). *Cron. Chim.*, No. 21 (1968) 25–30 and 48; *C.A.*, 72 (1970) 124981b.

WAGNER, J., HOFFMANN, H. AND LÖW, J.: Die Acylaglyka des Kryptoäscins und α-Äscins. *Z. Physiol. Chem.*, 351 (1970) 1133–1140.

WAGNER, J., SCHLEMMER, W. AND HOFFMANN, H.: Über Inhaltsstoffe des Rosskastaniensamens. IX. Struktur und Eigenschaften der Triterpenglykoside. *Arznzeim. Forsch.*, 20 (1970) 205–209.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

See PC section.

18. AMINO ACIDS

ENSOR, J. M. AND KENDALL-TAYLOR, P.: Comparison of paper and thin-layer radiochromatography for the quantitative analysis of thyroidal iodoamino acids. *J. Endocrinol.*, 46 (1970) 527–531; *C.A.*, 72 (1970) 118312t—TLC and PC.

GATTO, J. L. AND BORDERS, JR., C. L.: Separation of leucine and isoleucine by thin-layer chromatography. *J. Chem. Educ.*, 47 (1970) 840.

HAEMERS, A. AND DE MOERLOOSE, P.: The identification of polymyxin B sulphate. *J. Chromatogr.*, 52 (1970) 154–157.

KUTTER, D. AND HUMBEL, R.: Chromatographie sur couche mince des acides aminés dans les milieux biologiques. Application au dépistage et au diagnostic des aminoacidopathies. *Pharm. Acta Helv.*, 45 (1970) 553–563.

KWAPNIEWSKI, Z., DUK, B. AND SZTARK, W.: (Thin-layer chromatographic determination of amino acids using copper acetate and Variamine Blue). *Czas. Tech. (Krakow), M*, 2 (1969) 22–24; *C.A.*, 72 (1970) 129250x.

SKARSAUNE, S. K., YOUNGS, V. L. AND GILLES, K. A.: Changes in wheat lipids during seed maturation. II. Changes in lipid composition. *Cereal Chem.*, 47 (1970) 533–544.

TOMEO LACRUE, M. AND REVUELTA, D.: (Chromatographic determination of glutamic acid). *Rev. Acad. Cienc. Exactas, Fis.-Quim. Natur. Zaragoza*, 23 (1968) 207–213; *C.A.*, 72 (1970) 139473m.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

KEUTMANN, H. T., PARSON, J. A., POTTS, JR., J. T. AND SCHLUETER, R. J.: Isolation and chemical properties of two calcitonins from salmon ultimobranchial glands. *J. Biol. Chem.*, 245 (1970) 1491–1496.

19b. Elucidation of structure of proteins

DOMAGK, G. F., DOMSCHKE, W., MEYER, J. AND WEISE, M.: The N-terminal and C-terminal endgroups of glucose-6-phosphate dehydrogenase from *Candida utilis*. *Z. Physiol. Chem.*, 351 (1970) 923–926.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

ANTONELLO, R. C. AND CARLASSARE, F.: Isolation of a benzo[*a*]pyrene–thymine photoadduct from DNA hydrolyzed after irradiation at 365 nm in the presence of benzo[*a*]pyrene. *Z. Naturforsch.*, 25b (1970) 1269–1271.

GAUCHEL, F. D., BEYERMANN, K. AND ZAHN, R. K.: Microdetermination of DNA in biological materials by gas chromatographic and isotope dilution analysis of thymine content. *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 6 (1970) 141–144; *C.A.*, 72 (1970) 129251y.

HARRIS, A. B.: Rapid resolution of flavin-adenine dinucleotide and its mononucleotides on cellulose layers using aqueous ammonium salts and formic acid. *Biochim. Biophys. Acta*, 184 (1969) 467–469.

NELSON, D. L. AND KORNBERG, A.: Biochemical studies of bacterial sporulation and germination. XIX. Phosphate metabolism during sporulation. *J. Biol. Chem.*, 245 (1970) 1137–1145—cellulose impregnated with poly(ethyleneimine).

SHAW, G. AND SMALLWOOD, B. M.: Purines, pyrimidines and imidazoles. Part XXXIV. Synthesis and some reactions of N^6-(formylmethyl)adenine. *J. Chem. Soc. (C)*, (1970) 2206–2208.

SHEID, B. AND WILSON, S. M.: Inhibition of transfer RNA methylases by bull semen. I. Localization, identification and properties of the inhibitor. *Biochim. Biophys. Acta*, 224 (1970) 382–390.

UPTON, J. D.: Thin-layer chromatography of cyclic adenosine 3′, 5′-monophosphate on tetraborate-impregnated silica gel layers. *J. Chromatogr.*, 52 (1970) 171–172.

See also PC section.

22. ALKALOIDS

FIEBIG, A. M., FELCZAK, J. AND JANICKI, S.: (Chromatographic separation of tropane alkaloid degradation products). *Farm. Pol.*, 25 (1969) 971–972; *C.A.*, 73 (1970) 7134n.

GHARBO, S. A. AND HABIB, A. A. M.: Phytochemical investigation of Egyptian Senecio. II. Alkaloids of *Senecio aegyptius, S. desfontainei, S. vulgaris, S. petasitis* and *S. mikanioides*. *Lloydia*, 32 (1969) 503–508; *C.A.*, 73 (1970) 1132k.

STEPANENKO, O. B. AND SHEMYAKIN, F. M.: (Thin-layer chromatography of opium alkaloids performed on a buffered silica gel layer). *Farmatsiya (Moscow)*, 19, No. 1 (1970) 37–40; *C.A.*, 72 (1970) 136459a.

TULUS, R. AND ISKENDER, G.: (Combination of the method of Bamford with thin-layer chromatography in the analysis of alkaloids. II. Studies relating the groups 1–3). *Istanbul. Univ. Eczaclik Fak. Mecm.*, 5 (1969) 103–116; *C.A.*, 72 (1970) 125091y.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DOBRECKY, J.: (Analysis of vincaleucoblastine sulphate and *Podophyllum emodi* glucosides). *Rev. Asoc. Bioquim. Argent.*, 34 (1969) 91–94; *C.A.*, 73 (1970) 7286p.

PEYRE, J. PH. AND REYNIER, M.: Séparation, identification et dosage du pyrazole et de quelques dérivés 4-substitués. *Ann. Pharm. Fr.*, 27 (1969) 749–752.

SHEINKMAN, A. K. AND DEIKALO, A. A.: (Reaction of N-acylisoquinolinium salts with pyrroles). *Khim. Geterotsikl. Soedin.*, (1970) 126–127; *C.A.*, 72 (1970) 121327g.

24. ORGANIC SULPHUR COMPOUNDS

MATSUO, M.: New thin-layer chromatographic solvent systems for glucosinolates (mustard oil glucosides). *J. Chromatogr.*, 49 (1970) 323–324.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

KORIDZE, A. A. AND GUBIN, S. P.: *Cis-* and *trans-β*-substituted *α*-vinylferrocenes. Configuration of trisubstituted ethylenes. *J. Organometal. Chem.*, 22 (1970) 157–169; *C.A.*, 72 (1970) 132924a—R_F values for 16 compounds.

27. VITAMINS AND VARIOUS GROWTH FACTORS

BLATTNÁ, J., MANOUŠKOVÁ, J. AND DAVÍDEK, J.: (Chromatography of tocopherols). *Sb. Vys. Sk. Chem. Technol. Praze, Potraviny*, E 20 (1968) 19–26; *C.A.*, 72 (1970) 120218k.

KWAPNIEWSKI, Z., SZLENK, I. AND SLIWIOK, J.: (Separation of vitamin E and its oxidation products on modified silica gel). *Czas. Tech. (Krakow), M*, No. 2 (1969) 29–32.

SPINELLI, P. AND CIUFFI, M.: (Separation and determination of water-dispersed phytomenadione and tocopheryl acetate in a pharmaceutical preparation). *Boll. Chim. Farm.*, 108 (1969) 549–553; *C.A.*, 72 (1970) 125098f.

28. ANTIBIOTICS

ANONYMOUS: Griséofulvin. *Ann. Pharm. Fr.*, 27 (1969) 693–695.

SHAFER, H., VANDENHEUVEL, W. J. A., ORMOND, R., KUEHL, F. A. AND WOLF, F. J.: Characterization of phosphonomycin by microchromatographic and related techniques. *J. Chromatogr.*, 52 (1970) 111–117—TLC and PC.

29. INSECTICIDES AND OTHER PESTICIDES

GRUENE, A., NENDEL, K., PAHL, TH., SCHUBERT, K. AND WORF, G.: (Thin-layer chromatographic detection of pesticide residues in fruit and vegetables). *Riechst., Aromen, Koerperpflegem.*, 19 (1969) 494, 496, 498, 500, 550–554; *C.A.*, 72 (1970) 131197d.

KHMELEVSKII, B. N.: (Chromatographic determination of sevin in chicken eggs). *Tr. Vses. Nauch.-Issled. Inst. Vet. Sanit.*, 30 (1968) 232–235; *C.A.*, 72 (1970) 120362c.

30. SYNTHETIC AND NATURAL DYES

DIXON, E. J., GRISLEY, L. M. AND SAWYER, R.: The purification of solochrome cyanine R. *Analyst*, 95 (1970) 945–949—TLC and PC.

HORDYNSKA, S. AND LEGATOWA, B.: (Identification of dye bases and phenols in hair dyes by thin-layer chromatography). *Rocz. Panstw. Zakl. Hig.*, 20 (1969) 673–679; *C.A.*, 72 (1970) 124988j.

SHERMA, J.: Chromatography of fat-soluble chloroplast pigments on preformed kieselguhr layers. *J. Chromatogr.*, 52 (1970) 177–179.

ZVĚŘINA, V., DIVIŠ, J. AND MATRKA, M.: Analyse von Farbstoffen und von bei der Farbstofferzeugung anfallenden Zwischenprodukten. XII. Chromatographische Beurteilung der Reinheit von 1-aryl-3,3-dialkyltriazenverbindungen. *Coll. Czech. Chem. Commun.*, 35 (1970) 3109–3111.

See also PC section.

31. PLASTICS AND THEIR INTERMEDIATES

CAMPBELL, G. B., FOXTON, A. A. AND WORSDALL, R. L.: Identification of plasticizers in poly(vinylchloride) compounds by thin-layer chromatography. *Lab. Pract.*, 19 (1970) 369–371 and 373; *C.A.*, 72 (1970) 133448k—R_F values for 15 plasticizers.

VALK, G., LOERS, E. AND KUEPPERS, P.: (Role of oligomers in polyester textile manufacture). *Melliand Textilber.*, (1970) 504–508; *C.A.*, 72 (1970) 133966c.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

DEMIR, S. AND AMAL, H.: Analysis of pharmaceuticals containing antihistamines by quantitative thin-layer chromatography. *Istanbul Univ. Eczacilik Fak. Mecm.*, 5, No. 2 (1969) 80–92; *C.A.*, 72 (1970) 136508r.

EHRHARDT, L. AND SUCKER, H.: (Analytical procedure for detection of tablet and drug adjuvants. I. New methods for detecting individual adjuvants). *Pharm. Ind.*, 32 (1970) 92–97; *C.A.*, 73 (1970) 7294q—TLC and PC.

LE MOAN, G., ROUILLAC, R., JOLIVET, C. AND NIDRECOURT, A.: (Identification of meprobamate (procalmadiol) and of various drugs). *Ann. Fals. Expert. Chim.*, 62 (1969) 207–213; *C.A.*, 72 (1970) 125090x.

NAGASAWA, K., YOSHIDOME, H. AND ANRYU, K.: Separation and detection of synthetic sweetners by thin-layer chromatography. *J. Chromatogr.*, 52 (1970) 173–176.

NAKANO, K.: Incompatibility of commercial injections as examined by thin-layer chromatography. *Iryo (Tokyo)*, 23 (1969) 1441–1445; *C.A.*, 72 (1970) 136348p.

NOBREGA, P. AND NOBREGA, M. A. F.: (*m*-Aminophenol in *p*-aminosalicylic acid (PAS). Chromatographic separation and quantitative and semiquantitative analysis. Bromometry of PAS). *Rev. Brasil. Farm.*, 50 (1969) 129–137; *C.A.*, 73 (1970) 7333b.

PITEL, G. AND LUCE, TH.: Dosage du sulpiride en milieu biologique. *Ann. Pharm. Fr.*, 28 (1970) 409–415.

REISCH, J., KOMMERT, H. J. AND HESSE, U.: (Analysis of pharmaceuticals. 16. Detection of nitrogen-containing pharmaceuticals by photo colour reactions). *Pharm. Ztg.*, 114 (1969) 1968–1970; *C.A.*, 72 (1970) 114903p.

RÜMMLER, R. AND HALLER, R.: Chromatographische Trennungsmethoden für Methadon und die Reduktionsprodukte α- und β-Methadol. *Arzneimittelforsch.*, 20 (1970) 281–282.

SANDRI CAVICCHI, G., CRISTOFORI, B. AND QUAGLIO, M. P.: (Analytical character of antitussive drugs). *Boll. Chim. Farm.*, 108 (1969) 682–689; *C.A.*, 72 (1970) 136507q.

SIMMONS, D. L. AND RANZ, R. J.: Identification of incompatibility products between meprobamate and dichloroalphenazone. *Can. J. Pharm. Sci.*, 4, No. 4 (1969) 96; *C.A.*, 73 (1970) 7152s.

See also PC section.

32b. Metabolism of drugs, toxicological applications

BARBI, G., SARDINI, D., BASTONI, F., MARZO, A. AND TALLONE, G.: Thin-layer chromatography of urinary bromsulphalein metabolites in normal and cirrhotic subjects. *Biochim. Biol. Sper.*, 8 (1969) 149–155; *C.A.*, 72 (1970) 130321c.

HARRISON, A. J. AND COOK, A.: Case history of drug addiction and a thin-layer chromatographic system for the separation and identification of some drugs of addiction in sub-microgram amounts. *Forensic Sci. Soc. J.*, 9 (1969) 165–167; *C.A.*, 72 (1970) 136334f.

LAFARGUE, P., PONT, P. AND MEUNIER, J.: Étude des dérivés de la benzo[*f*]diazépine-1,4 utilisés en thérapeutique. I. Étude spectroscopique et par chromatographie en couche mince de ces dérivés, de leurs principaux métabolites et des composés formés par leur hydrolyse acide. *Ann. Pharm. Fr.*, 28 (1970) 343–354.

LE BOURHIS, B.: Identification de quelques métabolites du *trans*-anéthole chez l'homme, le lapin et le rat. *Ann. Pharm. Fr.*, 28 (1970) 355–361.

32c. Plant extracts

DANIELAK, R. AND BORKOWSKI, B.: Biologically active compounds in seeds of crucifers. III. Chromatographical search for glucosynolates. *Diss. Pharm. Pharmacol.*, 21 (1969) 563–575; *C.A.*, 73 (1970) 7140m.

KWIATKOWSKI, A. AND ROZMEJ, Z.: (Chromatographic methods of studying wood extracts. I. Method for examination of unsaponifiables). *Pr. Inst. Technol. Drewna*, 14, No. 2 (1967) 3–9; *C.A.*, 72 (1970) 123084z.

MATHIS, C.: (Identification of some plant tinctures (eucalyptus, vanilla, rhatany) by colour reactions and by thin-layer chromatography). *Bull. Soc. Pharm. Strasbourg*, 13 (1970) 35–39; *C.A.*, 72 (1970) 136491e.

WURMSER, L. AND VISCHNIAC, I.: Quelques observations sur la préparation des Teintures-Mères homoeopathiques. *Ann. Pharm. Fr.*, 28 (1970) 377–389.

See also TLC section 1.

33. INORGANIC SUBSTANCES

BABA, T., SAKUSHIMA, K. AND YONEDA, H.: Thin-layer chromatography of inorganic salts. III. Study of the hydration of anions by means of chromatography of Crystal violet cation. *Bull. Chem. Soc. Jap.*, 43 (1970) 931–932; *C.A.*, 72 (1970) 125621w.

CANIC, V. D., PETROVIC, S. M. AND PETROVIC, S.: (Separation of alkali metal (ions) on starch thin layers). *Glas. Hem. Drus., Beograd*, 33 (1968) 289–292; *C.A.*, 72 (1970) 136764w.

KWAPNIEWSKI, Z., OGIERMAN, L. AND SZTARK, W.: (Effect of the 2-component mobile phase on R_F and R_M values of phosphoric acid esters during thin-layer chromatography). *Czas. Tech. (Krakow), M*, No. 2 (1969) 28–29; *C.A.*, 72 (1970) 125366s.

MASSA, V.: (Photodensitometric determination of lithium in the presence of other alkaline metals following thin-layer chromatography). *Trav. Soc. Pharm. Montpellier*, 29 (1969) 209–212; *C.A.*, 72 (1970) 139246q.

VOLYNETS, M. P., ERMAKOV, A. N. AND NIKITINA, L. P.: (Thin-layer chromatography of precious elements. Separation of chloride complexes of precious metals by partition chromatography). *Zh. Anal. Khim.*, 25 (1970) 294–300; *C.A.*, 72 (1970) 139342t.

See also PC section.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35a. Surfactants

BRUNI, G. AND LOKAR, A.: (Analysis of non-ionic surfactants by thin-layer chromatography). *Univ. Studi Trieste, Fac. Econ. Commer. Ist. Merceol. (Pubbl.)*, No. 36 (1970) 23 pp.; *C.A.*, 72 (1970) 123276p.

35b. Antioxidants and preservatives

CHAKRAVARTI, S. N. AND KETTER, R.: (Thin-layer type chromatographic identification of phenolic antioxidants). *Kaut. Gummi, Kunstst.*, 23, No. 3 (1970) 93–95; *C.A.*, 72 (1970) 133823d.

Column Chromatography

1. REVIEWS AND BOOKS

BROWNING, D. R.: *Chromatography*, McGraw-Hill, New York, 1970, 151 pp.—an introduction containing fundamentals of column, gel permeation and ion-exchange chromatography.

DETERMANN, H.: Gel chromatography, a separatory method based on molecular-weight differences. *Chimia*, 23 (1969) 94–105; *Anal. Abstr.*, 19 (1970) 849.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

ELLINBOE, J., ALMÉ, B. AND SJÖVALL, J.: Introduction of specific groups into polysaccharide supports for liquid chromatography. *Acta Chim. Scand.*, 24 (1970) 463–467.

DE METS, AND LAGASSE, A.: Scanning electron micrographs of column packing. *J. Chromatogr. Sci.*, 8 (1970) 272–275—glass beads, Porapak, Sephadex, Bio-gel.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

MAJER, J. R., TRAVERS, S. AND WATSON, M.: Simple sensitive recording differential refractometer for column chromatography. *Talanta*, 16 (1969) 434–438; *Anal. Abstr.*, 18 (1970) 4441.

OLGE, D. G.: Chromatographic sample injection system. *Ger. Pat.*, Offen. 1, 915, 113, 23 Oct 1969; *C.A.*, 72 (1970) 28165t.

OWEN, J. A.: Range expansion in auto-analyser recorders. *Clin. Chim. Acta*, 29 (1970) 89–91.

SIEBKE, J. C. AND EKREN, T.: Chromatography of RNA–DNA complex on hydroxylapatite. A method for the separation of the complementary strands in T2 DNA. *Eur. J. Biochem.*, 12 (1970) 380–386—hydroxyapatite.

WALSH, K. A., MCDONALD, R. M. AND BRADSHAW, R. A.: Automatic systems for detecting cystine and cystinyl peptides during column chromatography. *Anal. Biochem.*, 35 (1970) 193–202—SE-Sephadex C-25, Dowex 50 X8.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

BESTMANN, H. J., ARMSEN, R. AND WAGNER, H.: Reaktionen mit Phosphinalkylenen. XXIV. Oxydation von Phosphinalkylenen mit Perjodat. Synthesemöglichkeiten für α, β-Dicarbonylverbindungen, Olefine und polycyclische Verbindungen. *Chem. Ber.*, 102 (1969) 2259–2269—alumina (act. II).

BESTMANN, H. J. AND KRAUZ, E.: Reaktionen mit Phosphinalkylenen. XXII. Eine neue Ringschlussreaktion. *Chem. Ber.*, 102 (1969) 1802–1815—alumina (act. I).

BROCKMANN, H. AND DICKE, F.: Die Konstitution des Dibenzo[*a,o*]perylen-Photooxides. *Chem. Ber.*, 103 (1970) 7–16—alumina (act. II).

HSIEH, B. C. B., WOOD, R. E., ANDERSON, L. L. AND HILL, G. R.: Fractionation of low-temperature coal tar by gel permeation chromatography. *Anal. Chem.*, 41 (1969) 1066–1073—Sephadex LH-20.

KRÜGER, K. AND LIPPERT, E.: Notiz über die säulenchromatographische *cis–trans*-Isomerentrennung von *meta*-Halogenstilbenen im präparativen Masstab. *Chem. Ber.*, 102 (1969) 3233–3234—alumina (act. I).

PAIONI, R. AND JENNY, W.: Höhere, kondensierte Ringsysteme. 5. Mitt. Makrocyclen mit überbrückten 9,10-Dihydrophenanthreneinheiten. *Helv. Chim. Acta*, 53 (1970) 141–154—alumina.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

ADITYACHANDHURY, N. AND GUPTA, P. K.: Flemichapparin B: a new pterocarpinoid from *Flemingia chappar* Ham. *Chem. Ind. (London)*, (1970) 745–746—silica gel.

TJUKAVKINA, N. A., BENEŠOVÁ, V. AND HEROUT, V.: Plant substances. XXXI. Flavonoid C-glycosides in liverwort, *Madotheca platyphylla* (L.) Dum. *Coll. Czech. Chem. Commun.*, 35 (1970) 1306–1308—polyamide.

9. OXO COMPOUNDS

NIWA, E. AND MIYAKE, M.: Notiz über den Bildungsmechanismus von 1,4-D-α-Furyl-butandion-(1,4) aus α-Furyllithium mit Sauerstoff. *Chem. Ber.*, 103 (1970) 997–998—silica gel.

NÓGRÁDI, M., FARKAS, L. AND OLLIS, W. D.: Notiz über die Synthese des Milldurons, eines Isoflavons aus *Millettia dura* (Dunn). *Chem. Ber.*, 103 (1970) 949–1000—silica gel.

10. CARBOHYDRATES

10a. Mono and oligosaccharides: structural studies

ČAPEK, K., ŠTEFFKOVÁ, J. AND JARÝ, J.: Amino sugars. XXII. Partial acetylation and deacetylation of derivatives of methyl 3-acetamido-3,6-dideoxy-α-D-gulopyranoside and methyl 3-acetamido-3,6-dideoxy-α-L-allopyranoside. *Coll. Czech. Chem. Commun.*, 35 (1970) 107–115—silica gel, neutral alumina.

IWASAKI, T. AND SCHMID, K.: Isolation and characterization of a 4 S α_2, β_1-glycoprotein of normal human plasma. *J. Biol. Chem.*, 245 (1970) 1814–1820—DEAE-Sephadex A-50, DEAE-cellulose, Sephadex G-100, hydroxyapatite.

JACKSON, R. L. AND HIRS, C. H. W.: The primary structure of porcine pancreatic ribonuclease. I. The distribution and sites of carbohydrate attachment. *J. Biol. Chem.*, 245 (1970) 624–636–Sephadex G-25, Sephadex G-50, SE-Sephadex C-50, Dowex 50 X2, 1 X2.

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

CHURMS, S. C., STEPHEN, A. M. AND VAN DER BIJL, P.: The effect of sample concentration on gel chromatography of polysaccharides on polyacrylamide gels. *J. Chromatogr.*, 47 (1970) 97–99—polyacrylamide gel, Bio-gel P-300, P-10.

HOTTA, K., HORIKAWA, M. AND KUROKAWA, M.: Purification and characterization of α_1-acid glycoprotein from ascitic fluid. *Anal. Biochem.*, 33 (1970) 463–469—CM-cellulose.

JANN, B., JANN, K. AND SCHMIDT, G.: Immunochemical studies of polysaccharide surface antigens of *Escherichia coli* 0100:K?(B):H_2. *Eur. J. Biochem.*, 15 (1970) 29–39—Sephadex G-50, G-100.

LI, Y.-T. AND LI, S.-C.: Isolation and characterization of α_1-acid glycoprotein from chimpanzee plasma. *J. Biol. Chem.*, 245 (1970) 825–832—CM-cellulose, Dowex 50 X8.

LISOWSKA, E.: The degradation of M and N blood group glycoproteins and glycopeptides with alkaline borohydride. *Eur. J. Biochem.*, 10 (1969) 574–579—Sephadex G-25, Bio-gel P-6, P-2.

MUIR, L. AND LEE, Y. C.: Glycopeptides from earthworm cuticle collagen. *J. Biol. Chem.*, 245 (1970) 502–509—Sephadex G-15, G-75, Bio-gel P-2, Sephadex LH-20.

PAULSEN, H. AND STEINERT, G.: Hydrazin-Reaktionen. IX. Cyclisierung von 5-Desoxy-5-(α-methylhydrazino)-D-xylose zum Siebenring-Hydrazon. *Chem. Ber.*, 103 (1970) 475–485—silica gel.

PUSZTAI, A. AND WATT, W. B.: Fractionation and characterization of glycoproteins containing hydroxyproline from the leaves of *Vicia faba*. *Eur. J. Biochem.*, 10 (1969) 523–532—Biogel P-100 or P-200, Sephadex G-75.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

LANOUE, K., NICKLAS, W. J. AND WILLIAMSON, J. R.: Control of citric acid cycle activity in rat heart mitochondria. *J. Biol. Chem.*, 245 (1970) 102–111—Dowex 1.

MOEHLER, K. AND PIRES, R.: (Separation of organic acids by column chromatography). *Z. Lebensm. Unters. Forsch.*, 139 (1969) 337–345; *Anal. Abstr.*, 19 (1970) 1752—Kieselgel.

SIMECHEN, G.: Reaktionen mit Halogenwasserstoffaddukten der Nitrile. III. Eine neue Pyridinsynthese. *Chem. Ber.*, 103 (1970) 389–397—Kieselgel.

11b. Lipids and their constituents

COUSINS, R. J., DE LUCA, H. F., SUDA, T., CHEN, T. AND TANAKA, Y.: Metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa. *Biochemistry*, 9 (1970) 1453–1459—silicic acid.

KATYAL, S. L., EAPEN, S. AND VENKITASUBRAMANIAM, T. A.: Fractionation of lipids on thin-layer plates, and its comparison with silicic acid column chromatography. *Indian J. Biochem.*, 6 (1969) 84–87; *Anal. Abstr.*, 19 (1970) 1550—silicic acid.

PURO, K.: Isolation of bovine kidney gangliosides. *Acta Chim. Scand.*, 24 (1970) 13–22—Anasil S.

11c. Lipoproteins

THIELE, O. W. AND KOCH, J.: The J blood group substance of cattle. J haptenic activity of glycosphingolipids of serum. *Eur. J. Biochem.*, 14 (1970) 37—silicic acid, Al_2O_3 (basic aluminium oxide).

13. STEROIDS

KOCH, H. J., SCHULZ, G. AND KIESLICH, K.: Mikrobiologische Reduktion von 3-Keto-19-nor-5(10)-en-steroiden zu entsprechenden 3-Hydroxy-Strukturen. *Chem. Ber.*, 103 (1970) 603–609—Kieselgel.

LAATIKAINEN, T. AND VIHKO, R.: Identification of $C_{19}O_2$ and $C_{21}O_2$ steroids in the glucuronide fraction of human bile. *Eur. J. Biochem.*, 10 (1969) 165–171—Sephadex LH-20.

MICKAN, H., DIXON, R. AND HOCHBERG, R. B.: Partition column chromatography of steroid triethylammonium salts. *Steroids*, 13 (1969) 477–482; *Anal. Abstr.*, 19 (1970) 1566—acid-washed Celite 545.

TOUCHSTONE, J. C., MURAWEC, T., KASPAROW, M. AND BALIN, A. K.: Quantitation of estriol in pregnancy urine by diazotic coupling and direct thin-layer densitometry. *J. Chromatogr. Sci.*, 8 (1970) 81–83.

TRAPP, G. A. AND WEST, C. D.: Determination of corticosteroid-binding proteins by an adsorption method. *J. Lab. Clin. Med.*, 73 (1969) 861–871; *Anal. Abstr.*, 19 (1970) 1606—Dextran-coated Florisil.

VAN TILBURG, A. M. J. AND MULLER, CH. J.: The influence of sodium chloride on the gel filtration of oestrogens on Sephadex. *Clin. Chim. Acta*, 29 (1970) 5–14—Sephadex G-15.

WIELAND, P. AND ANNER, G.: Über Steroide. 219. Mitt. Herstellung von 3-Oxo-17β-hydroxy-1,19-cyclo-5α-androstan. *Helv. Chim. Acta*, 53 (1970) 116–120—alumina, silica gel.

14. STEROID GLYCOSIDES AND SAPONINS

HAUSER, W., KARTNIK, T. AND VERDINO, G.: Contribution to the chemical evaluation of Digitalis drugs. X. *Digitalis lanata*. *Sci. Pharm.*, 37 (1969) 149–152; *Anal. Abstr.*, 19 (1970) 628—alumina.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

KŘEPINSKÝ, J., JOMMI, G., SAMEK, Z. AND ŠORM, F.: On terpenes. CCIV. The sesquiterpenic constituents of *Aristolchia debilis* Sieb. et Zucc. The structure of debilone. *Coll. Czech. Chem. Commun.*, 35 (1970) 745–748—SiO_2.

VOKÁČ, K., SAMEK, Z., HEROUT, V. AND ŠORM, F.: On terpenes. CCV. The structure of two native

orange substances from *Lactarius deliciosus* L. *Coll. Czech. Chem. Commun.*, 35 (1970) 1296–1301—silica gel, alumina.

VYSTRČIL, A. AND BUDĚŠÍNSKY, M.: Triterpenes. XVI. Unusual epimerisation of the $C_{(19)}$-acetyl group of 20-oxo-30-norlupane derivatives. *Coll. Czech. Chem. Commun.*, 35 (1970) 295–311—Al_2O_3, SiO_2.

VYSTRČIL, A. AND BUDĚŠÍNSKÝ, M.: Triterpenes. XVII. Preparation of new 19 αH-lupane derivatives. *Coll. Czech. Chem. Commun.*, 35 (1970) 312–320—alumina.

16. NITRO AND NITROSO COMPOUNDS

HORNER, L., ERTEL, I., RUPRECHT, H. D. AND BĚLOVSKÝ, O.: Phosphororganische Verbindungen. 64. Allyl–propenyl-Umlagerungen. Das elektrochemische Verhalten einiger Propenylverbindungen. *Chem. Ber.*, 103 (1970) 1582–1588—alumina.

LETSINGER, R. L. AND MCCAIN, J. H.: Photoreactions of nitroanisoles with cyanide ion. Studies of products and reaction sequence. *J. Amer. Chem. Soc.*, 91 (1969) 6425–6431—silica gel.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

DRELL, W.: Separation of catecholamines from catechol acids by alumina. *Anal. Biochem.*, 34 (1970) 142–151—alumina.

FUNASAKA, W., FUJIMURA, K. AND KURIYAMA, S. Studies of ligand-exchange chromatography. I. Separation of isomers of phenylenediamine. *Japan Analyst*, 18 (1969) 19–24; *Anal. Abstr.*, 19 (1970) 1403—Amberlite CG-120.

GONCI, D. A. AND PURDY, W. C.: Separation of diastereoisomers by chromatographic fractionation: resolution of optical isomers. *Separ. Sci.*, 4 (1969) 243–266; *Anal. Abstr.*, 19 (1970) 1327.

HARTKE, K. AND SALAMON, G.: Heterosubstituierte Fulvene. V. 5-Ring negativ substituierte 6,6-Diamino-fulvene. *Chem. Ber.*, 103 (1970) 147–155—alumina.

HATANO, H., SUMIZU, K., ROKUSHIKA, S. AND MURAKAMI, F.: Automatic liquid chromatography of primary mono- and diamines on a cation-exchange resin. *Anal. Biochem.*, 35 (1970) 377–383—Aminex A 4.

KIRMSE, W. AND EIGENWILLIG, G. G.: Desaminierung von 1,2-Diphenyläthylamin. *Chem. Ber.*, 103 (1970) 27–31—kieselguhr.

WENZEL, P.: Die thermische Spaltung des Tetraphenylhydrazins. Ein Beitrag zum Problem der nicht katalysierten Benzidin-Umlagerung. *Chem. Ber.*, 103 (1970) 1318–1333—silica gel.

WINTERFELD, E. AND NELKE, J. M.: Additionen an die Dreifachbindung. XIV. Kondensationen mit *o*-Amino-phenylessigsäure-Derivaten. *Chem. Ber.*, 103 (1970) 1183–1189—silica gel.

YOSHIOKA, M., OHARA, A., KONDO H. AND KANAZAWA, H.: Quantitative analysis of primary amines by ion-exchange chromatography. *Chem. Pharm. Bull. Tokyo*, 17 (1969) 1276–1279; *Anal. Abstr.*, 19 (1970) 1575—Aminex A-5.

18. AMINO ACIDS

CAVINS, J. F. AND FRIEDMAN. M.: An internal standard for amino acid analyses: S-β-(4-pyridylethyl)-L-cysteine. *Anal. Biochem.*, 35 (1970) 489–493—Hewlett-Packard Model 185 CHN analyser.

JACOBS, S.: An improved system for automatic amino acid analysis. *Analyst.* 95 (1970) 370–378.

PAIK, W. K. AND KIM, S.: ω-N-Methylarginine in protein. *J. Biol. Chem.*, 245 (1970) 88–92—Aminex A-5.

PETERSON, D. E. AND BERNLOHR, R. W.: Determination of muramic acid, ornithine and diaminopimelic acid during automatic amino acid analysis. *Anal. Biochem.*, 33 (1970) 238–243—Beckman PA 28 custom spherical resin, Bio-rad Aminex A-5.

ROLLAND, M., AQUARON, R. AND LISSITZKY, S.: Thyroglobulin iodoamino acids estimation after digestion with pronase and leucylaminopeptidase. *Anal. Biochem.*, 33 (1970) 307–317—Technicon amino acid analyser.

VENNEGOOR, G. AND BLOEMENDAL, H.: Effect of a purified post-microsomal fraction on amino acid incorporation *in vitro*. *Eur. J. Biochem.*, 15 (1970) 161–170—DEAE-Sephadex, Sephadex G-200.

VOELKERT, E. AND GRANT, D. R.: Determination of homoserine as the lactone. *Anal. Biochem.*, 34 (1970) 131–137—Aminex MS.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

JACKSON, R. L. AND HIRS, C. H. W.: The primary structure of porcine pancreatic ribonuclease.

II. The amino acid sequence of the reduced S-aminoethylated protein. *J. Biol. Chem.*, 245 (1970) 637–653—Dowex 50 X2, Sephadex G-25.

PHELAN, J. J. AND HIRS, C. H. W.: The primary structure of porcine pancreatic ribonuclease. III. The disulfide bonds. *J. Biol. Chem.*, 245 (1970) 654–661—Sephadex G-25.

SHOEI-YN LIN, ELLIS, H., WEISBLUM, B. AND GOODFRIEND, T.: Preparation and properties of iodinated angiotensins. *Biochem. Pharmacol.*, 19 (1970) 651–662—Dowex 50 X8.

WALL, R. A.: Separation of peptides on macroreticular ion exchangers. *Anal. Biochem.*, 35 (1970) 203–208—Amberlite IRA 900.

19b. Elucidation of structure of proteins

CHAVIN, S. I. AND FRANKLIN, E. C.: Studies on antigen-binding activity of macroglobulin antibody subunits and their enzymatic fragments. *J. Biol. Chem.*, 244 (1969) 1345–1352—Sephadex G-100, G-200, DEAE-cellulose.

DETONA, F. AND WESTLEY, J.: Active site peptides of rhodanese. *Biochim. Biophys. Acta*, 207 (1970) 144–149—Sephadex G-25, Bio-rad AG 1 X2.

GOETZL, E. J. AND METZGER, H.: Affinity labeling of a mouse myeloma protein which binds nitrophenyl ligands. Kinetics of labeling and isolation of a labeled peptide. *Biochemistry*, 9 (1970) 1267–1278—Sephadex G-50, QAE-Sephadex.

HEIDEMANN, E. AND HEINRICH, W.: Isolation and characterization of the peptides derived from the α_1-chain of calf skin collagen after hydroxylamine cleavage. *Eur. J. Biochem.*, 14 (1970) 61–69—CM-cellulose, Sephadex G-150.

HETTINGER, T. P. AND CRAIG, L. C.: Edeine. IV. Structure of the antibiotic peptides edeines A_1 and B_1. *Biochemistry*, 9 (1970) 1224–1232—Bio-rad AG 50 X4.

LEITZMANN, C., JANG-YEN WU AND BOYER, P. D.: Subunits, composition and related properties of succinyl coenzyme A synthetase. *Biochemistry*, 9 (1970) 2333–2346—Sephadex G-200, DEAE-Sephadex.

MEIGHEN, E. A. AND SCHACHMAN, H. K.: Hybridization of native and chemically modified enzymes. I. Development of a general method and its application to the study of the subunit structure of aldolase. *Biochemistry*, 9 (1970) 1163–1176—DEAE-Sephadex.

MONTGOMERY, P. C., BELLO, A. C. AND ROCKEY, J. H.: N-Terminal sequences of equine and human immunoglobulin heavy chains. *Biochim. Biophys. Acta*, 200 (1970) 258–266—Dowex 50X 2.

NEPOKROEFF, C. AND ARONSON, A. I.: Cell-free synthesis of ferredoxin in clostridial extracts. *Biochemistry*, 9 (1970) 2074–2081—Dowex 50 X2.

RILEY, W. D. AND SNELL, E. E.: Histidine decarboxylase of *Lactobacillus* 30a. V. Origin of enzyme-bound pyruvate and separation of non-identical subunits. *Biochemistry*, 9 (1970) 1485–1491—Sephadex G-25, DEAE-cellulose.

SANDERS, M. M., WALSH, K. A. AND ARNON, R.: Immunological cross-reaction between trypsin and chymotrypsin as a guide to structural homology. *Biochemistry*, 9 (1970) 2356–2363—Sephadex G-75.

SPIEGELBERG, H. L., PRAHL, J. W. AND GREY, H. M.: Structural studies of human γD myeloma protein. *Biochemistry*, 9 (1970) 2115–2127—Sephadex G-100.

20. PROTEINS (INCLUDING ENZYMES)

20a. Proteins of plant origin including bacteria

BRAUN, V. AND SIEGLIN, U.: The covalent murein–lipoprotein structure of the *Escherichia coli* cell wall. The attachment site of the lipoprotein on the murein. *Eur. J. Biochem.*, 13 (1970) 336–346—Dowex 50 X2.

FURLONG, C. E. AND WEINER, J. H.: Purification of a leucine-specific binding protein from *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, 38 (1970) 1076–1083—DEAE-cellulose.

20b. Plasma proteins

BOURGOIS, A. AND FOUGEREAU, M.: Isolation and characterization of the cyanogen bromide fragments of a mouse γG immunoglobulin. *Eur. J. Biochem.*, 12 (1970) 558–570—Sephadex G-100, G-75, G-50.

COHEN, I., BOHAK, Z., DE VRIES, A. AND KATCHALSKI, E.: Thrombosthenin M. Purification and interaction with thrombin. *Eur. J. Biochem.*, 10 (1969) 388–394—Sephadex G-200.

HALLER, W., TYMPNER, K. D. AND HANNING, K.: Preparation of immunoglobulin concentrates from human serum by chromatography on controlled pore glass. *Anal. Biochem.*, 35 (1970) 23–31—CPG, 174 A average size.

NOAT, G., RICARD, J., BOREL, M. AND GOT, C.: Étude directe par dialyse sur Sephadex G-25 de la formation d'un complexe entre l'hexokinase de levure et ses substrats. *Eur. J. Biochem.*, 11 (1969) 106–112—DEAE-cellulose, Sephadex G-100, G-25.

NOVOTNÝ, J., FRANĚK, F. AND ŠORM, F.: Large-scale isolation, characterization and classification

of pig immunoglobulin κ-chains. *Eur. J. Biochem.*, 14 (1970) 309–316—Sephadex G-50, SE-Sephadex C-25, Sephadex G-100.

WINTERSBERGER, U. AND WINTERSBERGER, E.: Studies on deoxyribonucleic acid polymerases from yeast. 2. Partial purification and characterization of mitochondrial DNA polymerase from wild type and respiration-deficient yeast cells. *Eur. J. Biochem.*, 13 (1970) 20–27—hydroxyapatite, DEAE-cellulose.

20c. Structural proteins

BOULANGER, P. A., FLAMENCOURT, P. AND BISERTE, G.: Isolation and comparative chemical study of structural proteins of the adenoviruses 2 and 5: hexon and fiber antigens. *Eur. J. Biochem.*, 10 (1969) 116–131—Sephadex G-200.

20e. Chromoproteins

PRICHARD, P. M., GILBERT, J. M., SHAFRITZ, D. A. AND ANDERSON, W. F.: Factors for the initiation of haemoglobin synthesis by rabbit reticulocyte ribosomes. *Nature*, 226 (1970) 511–514—Sephadex G-200.

WILSON, J. B., WRIGHTSTONE, R. N. AND HUISMAN, T. H. J.: Haemoglobin α chain duplication in barbary sheep, *Ammotragus lervia*, Pallas, 1777. *Nature*, 226 (1970) 354–355—CM-cellulose.

20g. Enzymes: oxidoreductases

DAVIDSON, B. E.: The identification of a reactive lysine residue in lobster glyceraldehyde-3-phosphate dehydrogenase. *Eur. J. Biochem.*, 14 (1970) 545–548—Sephadex G-50, G-25.

JÖRNVALL, H.: Horse liver alcohol dehydrogenase on the primary structure of an N-terminal part of the protein chain of the ethanol-active isoenzyme. *Eur. J. Biochem.*, 14 (1970) 521–534—Sephadex G-50.

JÖRNVALL, H. AND HARRIS, J. I.: Horse liver alcohol dehydrogenase on the primary structure of the ethanol-active isoenzyme. *Eur. J. Biochem.*, 13 (1970) 565–576—DEAE-cellulose, Sephadex G-50.

MILNE, P. R. AND WELLS, J. R. E.: Structural and molecular weight studies on the small copper protein, plastocyanin. *J. Biol. Chem.*, 245 (1970) 1566–1574—DEAE-cellulose, DEAE-Sephadex, Sephadex G-25, G-50, G-75.

MOFFA, D. J., LOTSPEICH, F. J. AND KRAUSE, R. F.: Preparation and properties of retinal-oxidizing enzyme from rat intestinal mucosa. *J. Biol. Chem.*, 245 (1970) 439–447—DEAE-cellulose, Sephadex G-200.

MÖRIKOFER-ZWEZ, S., CANTZ, M., KAUFMANN, H., VON WARTBURG, J. P. AND AEBI, H.: Heterogeneity of erythrocyte catalase correlations between sulfhydryl group content, chromatographic and electrophoretic properties. *Eur. J. Biochem.*, 11 (1969) 49–57—DEAE-cellulose, CM-cellulose.

PLAPP, B. V.: Enhancement of the activity of horse liver alcohol dehydrogenase by modification of amino groups at the active sites. *J. Biol. Chem.*, 245 (1970) 1727–1735—Sephadex G-25.

SANWAL, B. D.: Regulatory mechanisms involving nicotinamide adenine nucleotides as allosteric affectors. III. Control of glucose 6-phosphate dehydrogenase. *J. Biol. Chem.*, 245 (1970) 1626–1631—DEAE-cellulose.

20h. Enzymes: transferases

CANNATA, J. J. B.: Phosphoenolpyruvate carboxykinase from bakers' yeast. Isolation of the enzyme and study of its physical properties. *J. Biol. Chem.*, 245 (1970) 792–798—Sephadex G-200, DEAE-cellulose.

CHIRPICH, T. P., ZAPPIA, V., COSTILOW, R. N. AND BARKER, H. A.: Lysine 2,3-aminomutase. Purification and properties of a pyridoxal phosphate and S-adenosylmethionine-activated enzyme. *J. Biol. Chem.*, 245 (1970) 1778–1789—Sephadex G-100, DEAE-Sephadex A-50.

CONNETT, R. J. AND KIRSHNER, N.: Purification and properties of bovine phenylethanolamide N-methyltransferase. *J. Biol. Chem.*, 245 (1970) 329–334—Sephadex G-50, G-100, G-200, SE-Sephadex.

20i. Enzymes: hydrolases

BRESLOW, E. AND GIROTTI, A. W.: The interaction of ribonuclease with metal ions. III. Gel filtration studies on the relationship between cupric ion and cytidylic acid binding. *J. Biol. Chem.*, 245 (1970) 1527–1536—Sephadex G-25, Amberlite CG-50, CM-Sephadex G-50.

CUATRECASAS, P.: Topography of the active site of staphylococcal nuclease. Affinity labeling with diazonium substrate analogues. *J. Biol. Chem.*, 245 (1970) 574–584—Sepharose 4B.

FRITZ, H., GREIF, B., SCHRAMM, W., HOCHSTRASSER, K. AND WERLE, E.: Zur Identität des Polyvalenten Proteaseinhibitors aus Schafslunge mit dem Trypsin-Kallikrein-Inhibitor aus Rinderorganen. *Z. Physiol. Chem.*, 351 (1970) 139–144—Sephadex G-50, CM-cellulose.

FRITZ, H., SCHRAMM, W., GREIF, B., HOCHSTRASSER, K., FINK, E. AND WERLE, E.: Isolierung des Trypsininhibitors aus Schafspankreas. Verhalten gegenüber proteolytischen Enzymen. *Z. Physiol. Chem.*, 35 (1970) 145–150—Bio-gel P-2, Sephadex G-50, CM-cellulose.

SHIOKAWA, H. AND NODA, L.: Isolation and crystallization of acyl phosphatase from rabbit muscle. *J. Biol. Chem.*, 245 (1970) 669–673—CM-cellulose.

STROSBERG, A. D. AND KANAREK, L.: Immunochemical studies on hen's egg-white lysozyme. The role of the lysine, the histidine and the methionine residues. *Eur. J. Biochem.*, 14 (1970) 161–168—Amberlite IRC-50.

TOWLE, D. W. AND COPENHAVER, JR., J. H.: Partial purification of a soluble ($Na^+ + K^+$)-dependent ATPase from rabbit kidney. *Biochim. Biophys. Acta*, 203 (1970) 124–132—8% agarose.

TULSIANI, D. R. P. AND CARUBELLI, R.: Studies on the soluble and lysosomal neuraminidases of rat liver. *J. Biol. Chem.*, 245 (1970) 1821–1827—hydroxyapatite–cellulose.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

KANG, J. W., MOSELEY, K. AND MAITLIS, P. M.: Pentamethylcyclopentadienylrhodium and -iridium halides. I. Synthesis and properties. *J. Amer. Chem. Soc.*, 91 (1969) 5970–5977—Florisil.

NUGENT, M. J., CARTER, R. E. AND RICHARDS, J. H.: Metallocenyl carbonium ions. Participation by ferrocene in the solvolysis of β-ferrocenylalkyl tosylates. *J. Amer. Chem. Soc.*, 91 (1969) 6145–6151—alumina.

NUGENT, M. J., KUMMER, R. AND RICHARDS, J. H.: Metallocenyl carbonium ions. Stereochemistry of participation by ferrocene in the solvolysis of 2-ferrocenylalkyl tosylates. *J. Amer. Chem. Soc.*, 91 (1969) 6141–6145—alumina.

NUGENT, M. J. AND RICHARDS, J. H.: The stereospecific syntheses of ferrocene derivatives with leaving groups β to the metallocene. *J. Amer. Chem. Soc.*, 91 (1969) 6138–6140—alumina.

TSUTSUI, M., VELAPOLDI, R. A., SUZUKI, K., VOHWINKEL, F., ICHIKAWA, M. AND KOYANO, T.: Unusual metalloporphyrins. IV. Novel methods for metal insertion onto porphyrins. *J. Amer. Chem. Soc.*, 91 (1969) 6262–6266—alumina.

VAUGHAN, L. G. AND SHEPPARD, W. A.: New organogold chemistry. I. Synthesis and fluorine-19 nuclear magnetic resonance studies of some fluororganogold compounds. *J. Amer. Chem. Soc.*, 91 (1969) 6151–6156—Florisil.

28. ANTIBIOTICS

FELNER, I. AND SCHENKER, K.: Totalsynthese des Antibioticums Anisomycin. *Helv. Chim. Acta*, 53 (1970) 754–763—silica gel.

KELLER-SCHIERLEIN, W., SAUERBIER, J., VOGLER, U. AND ZÄHNER, H.: Stoffwechselprodukte von Mikroorganismen. 80. Mitt. Aranciamycin. *Helv. Chim. Acta*, 53 (1970) 779–789—Kieselgel.

ROTHWEILER, W. AND TAMM, C.: Isolierung und Struktur der Antibiotica Phomin und 5-Dehydrophomin. *Helv. Chim. Acta*, 53 (1970) 696–724—alumina, silica gel.

TZAGOLOFF, A.: Assembly of the mitochondrial membrane system. III. Function and synthesis of the oligomycin sensitivity-conferring protein of yeast mitochondria. *J. Biol. Chem.*, 245 (1970) 1545–1551—phosphocellulose.

Gas Chromatography

1. REVIEWS AND BOOKS

PERRY, S. G. AND GRANT, D. W.: Gas chromatography discussion group. *J. Chromatogr.*, 50 (1970) 544–546—report on the group meeting, London, April 1970.

STEVENS, M. P.: *Techniques and Methods of Polymer Analysis. Vol. 3. Characterization and Analysis of Polymers by Gas Chromatography*, Marcel Dekker, New York, 1969, 198 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

2c. Thermodynamics and theoretical relationships

KWA, T. L. AND BOELHOUWER, C.: Relations between gas chromatographic behaviour and chemical structure. III. The nature of internal rotation in methyl derivatives of benzene. *J. Chromatogr.*, 52 (1970) 21–29.

ZADO, F. M. AND FABECIC, J.: The physico-chemical fundamentals of gas chromatographic retention on porous polymer columns: Porapak Q and T. *J. Chromatogr.*, 51 (1970) 37–44.

2d. General

BAUDISCH, J., PAPENDICK, H.-D. AND SCHLOEDER, V.: Peakformen in der GLC in Abhängigkeit von Retentionszeit, Aufgabenmenge und Säulenart. *Chromatographia*, 3 (1970) 469–477.

BOČEK, P. AND NOVÁK, J.: Statistical processing of calibration data in quantitative analysis by gas chromatography. *J. Chromatogr.*, 51 (1970) 375–383.

PREMRU, L. AND KREMSER, M.: Gas chromatography on some self-associating octadecane derivatives. *J. Chromatogr.*, 51 (1970) 65–73.

TAKÁCS, J. AND KRÁLIK, D.: Contribution to the theory of the retention index system. II. Errors on the determination of the retention indices: the Erdey equation. *J. Chromatogr.*, 50 (1970) 379–384.

UHDEOVÁ, J.: Precision of gas chromatographic retention data in the manual processing of the chromatogram. *J. Chromatogr.*, 51 (1970) 23–36.

WIČAROVÁ, O., NOVÁK, J. AND JANÁK, J.: High precision measurement of specific retention volumes. *J. Chromatogr.*, 51 (1970) 3–11.

YANOVSKIĬ, M. Ts.: (Relation between retention volumes and refraction of some organic compounds). *Zh. Fiz. Khim.*, 44 (1970) 2451–2455.

3. TECHNIQUES I

3a. Detectors

CLEGG, J. B.: A solid electrolyte detector for the determination of trace amounts of oxygen in the presence of argon and other gases. *J. Chromatogr.*, 52 (1970) 367–374.

GERHARDT, K. O. AND AUE, W. A.: The negative alkali flame detector response. *J. Chromatogr.*, 52 (1970) 47–62.

MCWILLIAM, I. G.: A study of the flame ionisation detector. *J. Chromatogr.*, 51 (1970) 391–406.

NOVÁK, J., BOČEK, P., KEPRT, L. AND JANÁK, J.: The dependence of flame ionisation detector relative response factors on the configuration of the electrodes. *J. Chromatogr.*, 51 (1970) 385–390.

3b. Column performance and filling studies

BEREZKIN, V. G. AND FATEEVA, V. M.: (The influence of the surface of the solid support in gas–liquid chromatography). *Zh. Anal. Khim.*, 25 (1970) 2023–2027.

GOUGH, T. A. AND SIMPSON, C. F.: Variation of performance of porous polymer bead columns in gas chromatography. *J. Chromatogr.*, 51 (1970) 129–137.

NOVÁK, J.: Effects of the column length on the HETP in gas chromatography. *J. Chromatogr.*, 50 (1970) 385–390.

NOVÁK, J. AND BOČEK, P.: Pressure induced turbulence in a packed gas chromatographic column. *J. Chromatogr.*, 52 (1970) 221–225.

WOLF, F. AND LOSSE, A.: Zur Wirkung dünner Flüssigkeitsschichten auf Natriumchlorid und Mikroglaskugeln als Träger in der Gaschromatographie. *J. Chromatogr.*, 39 (1969) 475–481.

3c. Apparatus, accessories and materials for GC

ALIEV, M. I., KOZEĬKO, T. A. AND FISHER, S. I.: (Stationary liquid phases for gas–liquid chromatography). *Zh. Fiz. Khim.*, 44 (1970) 2413–2414.

BRAZHNIKOV, E. M., GODOVANNYĬ, V. D., RUSSIYAN, E. K. AND CHEREPNINA, O. O.: (Rotameters with small consumption of gas and liquid and coupled with valves of fine regulation). *Zh. Fiz. Khim.*, 44 (1970) 2665–2666—5–2000 ml of gas and 0.5–150 ml of liquid per minute.

CRONIN, D. A.: A simple quantitative method for trapping and transfer of low concentration gas chromatographic fractions suitable for use with small diameter glass column systems. *J. Chromatogr.*, 52 (1970) 375–383.

DUFKA, O., MALINSKÝ, J., CHURÁČEK, J. AND KOMÁREK, K.: "Synachrom", a macroporous copolymer, as a sorption material for gas chromatography. *J. Chromatogr.*, 51 (1970) 111–117.

NOVÁK, J. AND BOČEK, P.: Experimental study of the effect of column length and pressure on the HETP in gas chromatography. *J. Chromatogr.*, 52 (1970) 1–7.

VÍŠKA, J., KISS, F., POLLAK, M. AND POSPÍCHAL, O.: Properties of acid-washed and silanized Czechoslovak supports: Chromaton N and Chezasorb. *J. Chromatogr.*, 51 (1970) 103–110.

4. TECHNIQUES II

4a. Preparative-scale GC

AYERS, G. S., MOSSHOLDER, J. H. AND MONROE, R. E.: Preparative gas chromatographic resolution of 3,3-dimethyl-2-butanol and other alcohols. *J. Chromatogr.*, 51 (1970) 407–414.

4b. Programmed temperature and programmed pressure GC

GUERMOUCHE, M. H., FATSCHER, M. AND VERGNAUD, J.-M.: Étude des paramètres de la chromatographie en phase gazeuse réalisée en couplant un gradient longitudinal et une programmation de température. *J. Chromatogr.*, 52 (1970) 9–20.

4d. Special microtechniques and functional analysis

ARMENANTE, M., SANTORO, V. AND SPADACCINI, G.: Radiolysis products as reference substances in gas chromatography. *J. Chromatogr.*, 52 (1970) 227–235.

HARRISON, L. G. AND KOGA, Y.: A second-order reaction on a gas chromatographic column. II. Decomposition of IBr on a chromatographic column. *J. Chromatogr.*, 52 (1970) 39–45.

HARRISON, L. G., KOGA, Y. AND MADDEROM, P.: A second-order reaction on a gas chromatographic column. I. Computational analysis by the plate theory. *J. Chromatogr.*, 52 (1970) 31–38.

MAIER, H. G.: Eine Fehlermöglichkeit bei der Kopfraumanalyse. *J. Chromatogr.*, 50 (1970) 329–331.

4e. Automation

GAINKO, N. K. AND DOROGOCHINSKIĬ, A. Z.: (Separation of propylene from the propane–propylene fraction by means of continuous adsorption on zeolite). *Khim. Tekhnol. Topliva i Masel*, 56, No. 9 (1970) 9–12.

4f. Measurement of physico-chemical and related values

ANDREEV, L. V., BAĬBURSKIĬ, V. L., LAPTEVA, F. I. AND KUVSHINNIKOV, V. D.: (Impulse displacement methods of gas chromatography and their application to the study of adsorption and catalysis. I. Impulse-impulse displacement technique). *Zh. Fiz. Khim.*, 44 (1970) 2350–2353.

BELYAKOVA, L. D., KEĬBAL, V. L. AND KISELEV, A. V.: (Gas chromatographic investigation of adsorption of carbon dioxide on zeolite of the NaX type). *Zh. Fiz. Khim.*, 44 (1970) 2345–2349.

DOLLIMORE, D., HEAL, G. R. AND MARTIN, D. R.: An improvement in the elution technique for measurement of adsorption isotherms by gas chromatography. *J. Chromatogr.*, 50 (1970) 209–218.

GOLOVNYA, R. V. AND ARSENYEV, YU. N.: Gas chromatographic method for determination of enthalpy of solution from retention indices. *Chromatographia*, 3 (1970) 455–461.

RUDENKO, B. A., YUFIT, S. S., KRASNAYA, ZH. A. AND KUCHEROV, V. F.: (Determination of thermodynamic parameters of dissolution of some unstable compounds by gas chromatography). *Izv. Akad. Nauk. SSSR, Ser. Khim.*, 35 (1970) 2199–2207.

SHAPOSHNIKOV, YU. K. AND LIKHOTKIN, E. S.: (Study on the solubility of some organic substances in N-methylpyrrolidone by gas chromatography). *Neftekhimiya*, 10 (1970) 616–619.

SHEVCHUK, I. M., BOGOSLOVSKIĬ, YU. N. AND SAKHAROV, V. M.: (Calculation of the chromatographic characteristics of substances on stationary phases—members of a homologous series). *Zh. Anal. Khim.*, 25 (1970) 2018–2022.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5b. Other hydrocarbons

ALIEV, M. I., KOZEĬKO, T. A. AND FISHER, S. I.: (Capillary chromatography of C_8–C_{12} alkylbenzenes and the dependence of their retention volumes on their boiling points). *Khim. Tekhnol. Topliva i Masel*, 56, No. 10 (1970) 47–51.

BEBRIS, N. K., GIENKO, E. YA., ZAĬTSEVA, G. E., KISELEV, A. V., KUSTOVA, G. L., LIPKIND, B. A., NIKITIN, YU. S. AND YASHIN, YA. I.: (Gas chromatography of hydrocarbons and their derivatives on Silochrom). *Neftekhimiya*, 10 (1970) 773–777.

BEREZKIN, V. G., GROMOVA, G. L., GRIKINA, O. E. AND YAROVOĬ, S. S.: (Calculation of the retention times of olefins in gas–liquid chromatography). *Neftekhimiya*, 10 (1970) 754–762—theory of the method described.

CRAMERS, C. A., RIJKS, J. A., PACÁKOVÁ, V. AND DE ANDRADE, I. R.: The application of precision gas chromatography to the identification of types of hydrocarbons. *J. Chromatogr.*, 51 (1970) 13–21.

DOROKHOV, A. P., EMEL'YANOVA, G. V., IOFFE, I. I., MEL'NIKOVA, N. P. AND SHEFTER, V. E.: (The application of two-stage project of gas chromatographic analysis of unstable catalysis products from reforming). *Khim. Tekhnol. Topliva i Masel*, 56, No. 9 (1970) 53–55.

KRUPČÍK, J., LIŠKA, O. AND SOJÁK, L.: The applicability of the linear free energy relationships for the identification of some alkyl aromatic hydrocarbons by open tubular column gas chromatography. *J. Chromatogr.*, 51 (1970) 119–127.

KUGUCHEVA, E. E. AND ALEKSEEVA, A. V.: (Microcatalytic method of identification of diene hydrocarbons in gas chromatography). *Neftekhimiya*, 10 (1970) 778–783.

KURAŠ, M. AND HÁLA, S.: The use of a gas chromatograph–mass spectrometer for the analysis of complex hydrocarbon mixtures. *J. Chromatogr.*, 51 (1970) 45–57.

MARTYNOV, A. A. AND VIGDERGAUZ, M. S.: (The calculation of retention characteristics in gas–liquid chromatography of isomeric paraffins). *Neftekhimiya*, 10 (1970) 763–772.

ŠIMEKOVÁ, J., PRONAYOVÁ, N., PIEŠ, R. AND ČIHA, M.: Chromatographic analysis of primary light gasoline and Pbi-H fractions of Romashkino crude oil. *J. Chromatogr.*, 51 (1970) 91–101.

SOJÁK, L. AND BUČINSKÁ, A.: Open tubular column gas chromatography of dehydrogenation products of C_6–C_{10} *n*-alkanes. Separation and identification of mixtures of C_6–C_{10} straight-chain alkanes, alkenes and aromatics. *J. Chromatogr.*, 51 (1970) 75–82.

VANĚK, J., PODROUŽKOVÁ, B. AND LANDA, S.: Gas chromatographic behaviour of tricyclic saturated hydrocarbons. The relation between the retention indices and the stereochemical structure of the tricyclanes. *J. Chromatogr.*, 52 (1970) 77–86.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

ANDERSEN, R. A. AND VAUGHN, T. H.: Rapid electron capture determination of caffeic acid and quercetin moieties in plants. *J. Chromatogr.*, 52 (1970) 385–392.

9. OXO COMPOUNDS

GRUBER, H. L. AND PLAINER, H.: Gas-chromatographische Analyse von Formaldehyd und seinen Beiprodukten. *Chromatographia*, 3 (1970) 490–496.

MYSAK, A. E., VERETENOVA, T. N., ZAKUPRA, V. A., LEBEDEV, E. V. AND PLIEV, T. N.: (Analysis of 1,2-epoxyalkanes by gas chromatography). *Neftekhimiya*, 10 (1970) 608–615—no reference to a paper thematically very closely related to the topic (*e.g.*, V. ULBRICH AND O. DUFKA, *Chem. Prum.*, 10 (1960) 549).

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

ALLEN, I. D. AND HAKEN, J. K.: Gas chromatography of homologous esters. Part IV. Influence of stationary phase polarity on retention of unsaturated esters. *J. Chromatogr.*, 51 (1970) 415–422.

DE LINDEMANN, L.: Separation of mono- and dibasic fatty acids by gas chromatography. *J. Chromatogr.*, 51 (1970) 297–300.

MYSAK, A. E., ZAKUPRA, V. A., PIVOVAROVA, T. E., LEBEDEV, E. V., PLIEV, T. N. AND ZAIKA, T. D.: (Gas chromatographic analysis of branched carboxylic acids formed during the carboxylation of C_6–C_{10} α-alkenes). *Zh. Anal. Khim.*, 25 (1970) 2014–2017.

STARUSZKIEWICZ, JR., W. F., BOND, J. F. AND SALWIN, H.: Quantitative gas chromatographic determination of β-hydroxybutyric acid with application to eggs. *J. Chromatogr.*, 51 (1970) 423–432.

STOLL, U.: Die Kombination von Dünnschicht- und Gaschromatographie bei der Identifizierung aliphatischer Hydroxy- und Dicarbonsäuren. *J. Chromatogr.*, 52 (1970) 145–151.

UMEH, E. O.: Separation and determination of low-molecular-weight straight-chain (C_1–C_8) carboxylic acids by gas chromatography of their anilide derivatives. *J. Chromatogr.*, 51 (1970) 147–154.

UMEH, E. O.: Determination of formic acid in dilute aqueous solution and separation and determination of aniline and toluidine isomers by gas chromatography of formanilide and formyl toluidides. *J. Chromatogr.*, 51 (1970) 139–146.

WATSON, J. R. AND CRESCUOLO, P.: Gas chromatographic separation and detection of C_1 to C_3 monocarboxylic acids as the *p*-substituted benzyl esters. *J. Chromatogr.*, 52 (1970) 63–67.

ZHUKOV, A. V. AND VERESHCHAGIN, A. G.: Heptadecenoic acid as an internal standard in the gas chromatographic weight determination of fatty acids. *J. Chromatogr.*, 51 (1970) 155–166.

11b. Lipids and their constituents

MCCULLAGH, K. G., LINCOLN, H. G. AND SOUTHGATE, D. A. T.: Fatty acid composition of milk fat of the African elephant. *Nature*, 222 (1969) 493–494.

13. STEROIDS

CHALLIS, J. R. G. AND HEAP, R. B.: Steroid mono- and diheptafluorobutyrates; preparation, purification and estimation. *J. Chromatogr.*, 50 (1970) 228–238.

JUNG, G., BREITMAIER, E. AND VOELTER, W.: Analyse von Hydroxysteroiden mit Hilfe der ^{19}F-NMR-Spektroskopie. Strukturbestimmungen und quantitative Analysen von Hydroxysteroidgemischen im Mikrogrammbereich. *Z. Anal. Chem.*, 252 (1970) 304–306—GC–NMR.

NAMBARA, T., IWATA, T. AND HONMA, S.: Analytical chemical studies on steroids. XXXVII. Gas chromatography of 2,3-oxygenated estratrienes. *J. Chromatogr.*, 50 (1970) 400–404.
NOCKE, L.: Methode zur gleichzeitigen Bestimmung von Pregnandiol und Pregnantriol aus einer Urinprobe. *Z. Anal. Chem.*, 252 (1970) 322–327.
SIEKMANN, L., HOPPEN, H.-O. AND BREUER, H.: Zur gas-chromatographisch–massenspektrometrischen Bestimmung von Steroidhormonen in Körperflüssigkeiten unter Verwendung eines Multiple Ion Detectors (Fragmentographie). *Z. Anal. Chem.*, 252 (1970) 294–298.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

HEMINGWAY, R. W., HILLIS, W. E. AND BRUERTON, K.: A gas–liquid chromatographic examination of stilbene derivatives. *J. Chromatogr.*, 50 (1970) 391–399.
MACDONALD, B. F. AND SWAN, E. P.: Chromatography of the lignans of *Thuja plicata* Donn. *J. Chromatogr.*, 51 (1970) 553–555.

16. NITRO AND NITROSO COMPOUNDS

DALTON, R. W., KOHLBECK, J. A. AND BOLLETER, W. T.: Gas chromatographic analysis of trinitrotoluene from the continuous process. *J. Chromatogr.*, 50 (1970) 219–227.
HOFFSOMMER, J. C.: Quantitative analysis of nitro compounds in the micro- to picogram range by a combination of thin-layer and vapor phase chromatography with the nickel-63 electron capture detector. *J. Chromatogr.*, 51 (1970) 243–251.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

NAHRSTEDT, A.: GLC diastereomerer Benzaldehydcyanhydringlycoside. *J. Chromatogr.*, 50 (1970) 518–520.
SEN, N. P.: Gas–liquid chromatographic determination of dimethylnitrosamine as dimethylnitramine at picogram levels. *J. Chromatogr.*, 51 (1970) 301–304.

18. AMINO ACIDS

PARR, W., YANG, C., PLETERSKI, J. AND BAYER, E.: Rapid gas chromatographic separation of amino acid enantiomers using N-perfluoroacyl esters. *J. Chromatogr.*, 50 (1970) 510–512.
VANDENHEUVEL, W. J. A., SMITH, J. L., PUTTER, I. AND COHEN, J. S.: Gas–liquid chromatography and mass spectrometry of deuterium-containing amino acids as their trimethylsilyl derivatives. *J. Chromatogr.*, 50 (1970) 405–412.
VOLPERT, E. M., KUNDU, N. AND DAWIDZIK, J. B.: N_2O-Dipivalyl methyl esters of the thyroid hormones and related compounds: Synthesis, purification and gas chromatography. *J. Chromatogr.*, 50 (1970) 507–510.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19b. Elucidation of structure of proteins

ROACH, D. AND GEHRKE, C. W.: The hydrolysis of proteins. *J. Chromatogr.*, 52 (1970) 393–404.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

GAUCHEL, F. D., BEYERMANN, K. AND ZAHN, R. K.: Gas-chromatographische Bestimmung des DNS-Gehaltes biologischen Materials über den Thymingehalt. *Z. Anal. Chem.*, 252 (1970) 331–331.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

FLINT, G. T. AND AUE, W. A.: The gas–liquid chromatographic determination of 2-hydroxy-*s*-triazines. *J. Chromatogr.*, 52 (1970) 487–490.
GRUNWALD, C. AND LOCKARD, R. G.: Analysis of indole acid derivatives by gas chromatography using liquid phase OV-101. *J. Chromatogr.*, 52 (1970) 491–493.

24. ORGANIC SULPHUR COMPOUNDS

CARTONI, G. P., LIBERTI, A. AND ZOCCOLILLO, L.: The gas chromatographic behaviour of some isomeric derivatives of thiophene and furan. *J. Chromatogr.*, 52 (1970) 347–350.
MARTINŮ, V. AND JANÁK, J.: Gas–liquid chromatographic retention data of some aliphatic and alicyclic sulphides. *J. Chromatogr.*, 52 (1970) 69–75.
NIKITINA, V. S., LYAPINA, N. K. AND ULENDEEVA, A. D.: (Separation of sulphides from sulphur-containing petroleum condensates). *Neftekhimiya*, 10 (1970) 594–598.

PRILEZHAEVA, E. N., SHEKHTMAN, R. I., RUDENKO, B. A., SNEGOTSKIĬ, V. I. AND LABA, V. I.: (Application of gas chromatography to the separation of some aliphatic unsaturated sulphur-containing compounds). *Izv. Akad. Nauk SSSR, Ser. Khim.*, 35 (1970) 1945–1949.

27. VITAMINS AND VARIOUS GROWTH FACTORS

PANALAKS, T.: A gas chromatographic method for the determination of vitamin D in fortified non-fat dried milk. *Analyst*, 95 (1970) 862–867.

28. ANTIBIOTICS

SHAFER, H., VANDENHEUVEL, W. J. A., ORMOND, R., KUEHL, F. A. AND WOLF, F. J.: Characterization of phosphonomycin by microchromatographic and related techniques. *J. Chromatogr.*, 52 (1970) 111–117.

29. INSECTICIDES AND OTHER PESTICIDES

COHA, F. AND NEDIC, M.: An improved gas–liquid chromatographic method for the analysis of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT) and its metabolites in milk. *Anal. Lett.*, 3 (1970) 419–425.

FISHBEIN, L. AND ALBRO, P. W.: Thin-layer and gas–liquid chromatography of derivatives of isomeric chlorophenols. Chlorophenyl fluorosulphonyl-benzenesulfonates and related compounds. *J. Chromatogr.*, 51 (1970) 219–226.

JAGLAN, P. S. AND GUNTHER, F. A.: Effect of liquid phase loading and temperature on the relative retentions in the gas chromatography of methyl parathion and parathion. *J. Chromatogr.*, 50 (1970) 520–522.

RIVERS, J. B., YAUGER, JR., W. L. AND KLEMMER, H. W.: Simultaneous gas chromatographic determination of 2,4-D and dicamba in human blood and urine. *J. Chromatogr.*, 50 (1970) 334–337.

TAYLOR, I. S.: Design of a column for the gas chromatographic analysis of chlorinated hydrocarbon pesticides. *J. Chromatogr.*, 52 (1970) 141–144.

31. PLASTICS AND THEIR INTERMEDIATES

DEUR-ŠIFTAR, Đ. AND ŠVOP, V.: Characterization of polypropylene by pyrolysis gas chromatography. *J. Chromatogr.*, 51 (1970) 59–64.

KUBÁT, J., ŠIMÁNEK, J. AND ZACHOVAL, J.: Gas chromatography of the products formed by thermal decomposition of "pyrolysis resin". *J. Chromatogr.*, 51 (1970) 83–89.

YASUDA, S. K.: Determination of trace amounts of Eastman 910 adhesive in certain plastic-bonded explosives. *J. Chromatogr.*, 51 (1970) 261–265.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

CASSELMAN, A. A. AND BANNARD, R. A. B.: Gas-liquid chromatography of Caramiphen hydrochloride and its analogues. *J. Chromatogr.*, 52 (1970) 138–140.

DONIKE, M., JAENICKE, L., STRATMANN, D. AND HOLLMANN, W.: Gaschromatographischer Nachweis von stickstoffhaltigen Pharmaka in wässrigen Lösungen mit dem Stickstoffdetektor. *J. Chromatogr.*, 52 (1970) 237–250.

32b. Metabolism of drugs, toxicological applications

MCAULEY, F. AND KOFOED, J.: Preparative gas chromatography as a clean-up procedure in toxicology. *J. Chromatogr.*, 50 (1970) 513–517.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

CHURÁČEK, J., KOMÁRKOVÁ, H., KOMÁREK, K. AND DUFKA, O.: Gaschromatographische Trennung eines Gemisches von Edelgasen und Permanentgasen und von niedrigeren gesättigten und ungesättigten Kohlenwasserstoffen. *Chromatographia*, 3 (1970) 456–468.

33b. Volatile inorganic substances

MILESHKEVICH, V. P., NIKOLAEV, G. A. AND KARLIN, A. V.: (Quantitative determination of water by gas–liquid chromatography). *Zh. Anal. Khim.*, 25 (1970) 1978–1981.

NERSESYANTS, A. B., ZAKHAROV, E. L. AND BYSTROVA, Z. A.: (Gas chromatographic determination of aluminium and aluminium carbide). *Zavodsk. Lab.*, 36 (1970) 1043–1044—as H_2 and CH_4 after HCl digestion.

PARISSAKIS, G., VRANTI-PISCOU, D. AND KONTOVANNAKOS, J.: Gas chromatography of inorganic volatile chlorides. A study of their reactivity towards certain column packing materials. *J. Chromatogr.*, 52 (1970) 461–468.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

CYMBALUK, N. F. AND NEUDOERFFER, T. S.: A quantitative gas–liquid chromatographic determination of aromatic aldehydes and acids from nitrobenzene oxidation of lignin. *J. Chromatogr.*, 51 (1970) 167–174.

KAZINIK, E. M.: (Gas chromatography of some diphenyl oxide and diphenyl sulphone derivatives). *Zh. Anal. Khim.*, 25 (1970) 2054–2056.

KLEMM, L. H., SHABTAI, J. AND LEE, F. H. W.: Gas chromatography of some nitrogen and sulfur heterocycles by means of silicone and bentone–silicone phases. *J. Chromatogr.*, 51 (1970) 433–439.

TUNSTALL, F. I. H. AND CARVER, F. W. S.: The determination of residual solvents in propellant compositions by gas chromatography. *Chromatographia*, 3 (1970) 462–464.

Electrophoretic Techniques

1. REVIEWS AND BOOKS

HEIMBURGER, N. AND SCHWICK, H. G.: Characterization and isolation of proteins by electrophoretic methods. *Chromatogr. Electrophor. Symp. Int., 4th, 1966*, (Pub. 1968) 39–61; *C.A.*, 72 (1970) 86952h.

PASTUSKA, G. AND KRUEGER, R.: New developments in thin-layer electrophoresis. *Chromatogr. Electrophor. Symp. Int., 4th, 1966*, (Pub. 1968) 180–186; *C.A.*, 72 (1970) 87014r—thin-layer electrophoresis.

PREETZ, W.: Ionophoresis separation procedures in analytical chemistry. *Fortschr. Chem. Forsch.*, 11 (1969) 375–429; *Anal. Abstr.*, 18 (1970) 4471.

PRISTOUPIL, T. I.: (Micro-chromatography and micro-electrophoresis on nitrocellulose membranes). *Chem. Listy*, 63 (1969) 577–595; *Anal. Abstr.*, 19 (1970) 1852—nitrocellulose.

SARGENT, J. R.: *Methods of Zone Electrophoresis*, 2nd ed., British Drug House Chemicals, Poole, England, 1969, 118 pp.; *C.A.*, 72 (1970) 136821n.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

ABADI, D. M.: Discontinuous electrophoresis on polyacrylamide gels. *Lab. Clin. Chem.*, 15 (1969) 35–41; *Anal. Abstr.*, 18 (1970) 2886—polyacrylamide gel.

ABATUROVA, N. A.: (Porous fibrous condensation structures of cellulose acetate). *Khim. Tekhnol. Proizvod. Tselliul.*, (1968) 279–288; *C.A.*, 72 (1970) 134349r.

AWDEH AND ZUHAYR, L.: Straining method for proteins after isoelectric focusing in polyacrylamide gel. *Sci.Tools*, 16 (1969) 42–43; *C.A.*, 72 (1970) 107683p—isoelectric focusing in polyacrylamide gel.

KALASHNIKOV, V. P. AND KLYUEVA, S. G.: (Electrophoresis on agar gel with a system for flowing buffer solution). *Lab. Delo*, (1968) 756–757; *Anal. Abstr.*, 18 (1970) 2105.

LÁSZLÓ, T.: (Apparatus for paper and gel electrophoresis). *Revta Chim.*, 19 (1968) 545–546; *Anal. Abstr.*, 18 (1970) 686.

MITTELBACH, F., ANTL, W. AND HAMBURGER, K.: (Modification of disc electrophoresis for direct electrophoresis of frozen tissue sections). *Z. Klin. Chem. Klin. Biochem.*, 7 (1969) 189–193; *Anal. Abstr.*, 19 (1970) 545.

PHILPOT, J. S.: Electrophoretic separation apparatus. *Brit. Pat.*, 1,186,184 (Cl. 301*K*), 02 Apr 1970; Appl. 23 March 1966; 13 pp.; *C.A.*, 72 (1970) 134513q.

PRZYBYLA, H. AND KWAPNIEWSKI, Z.: (Multi-purpose apparatus for paper and for thin-layer electrophoresis. I). *Zesz. Nauk. Politech. Krakow, Chem.*, (1968) 3–11; *C.A.*, 72 (1970) 134476e.

RILBE, H. AND PETTERSON, S.: Simple method for preparation of approximately constant density in small columns. *Separ. Sci.*, 3 (1968) 535–549; *Anal. Abstr.*, 18 (1970) 2106.

WANG, K.-T. AND WU, P.-H.: Polyamide-layer chromatography. XV. Apparatus for electrophoresis. *Chemistry (Taipei)*, (1968) 83–85; *Anal. Abstr.*, 17 (1969) 2477—acryl resin plates.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

TLACH, K. F.: (Universal separation chamber for zone electrophoresis). *Jena Rev.*, 14 (1969) 139–141; *Anal. Abstr.*, 19 (1970) 907.

VIRTANEN, R. AND KIVALO, P.: New quantitative high-voltage zone electrophoresis method. *Acta Chem. Fenn.*, 42 (1969) 182–184; *Anal. Abstr.*, 19 (1970) 910.

WACHTER, H.: Automatic staining apparatus for electrophoretic media. *G-I-T*, 12 (1968) 1174, 1177–1178; *Anal. Abstr.*, 18 (1970) 2112.

7. PHENOLS

WALKER, J. R. L. AND THOMPSON, J. E.: Thin-layer electrophoresis of phenolic compounds. *Lab. Pract.*, 18 (1969) 629–631, 653; *Anal. Abstr.*, 19 (1970) 1549—Whatman CC41 cellulose.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BURKHARDT, R.: Electrophoresis of tannin substance of wine with use of a gelatinised cellulose acetate film. *Nahrung*, 12 (1968) 615–621; *Anal. Abstr.*, 18 (1970) 584—gelatinised cellulose acetate.

10. CARBOHYDRATES

10a. Mono and oligosaccharides: structural studies

FRAHN, J. L.: Limitations of some common indicators of electroendosmosis in paper electrophoresis (including interactions between carbohydrates and inorganic phosphate). *J. Chromatogr.*, 48 (1970) 577–580—Whatman No. 4 paper.

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

DUDMAN, W. P. AND BISHOP, C. T.: Electrophoresis of dyed polysaccharides on cellulose acetate. *Can. J. Chem.*, 46 (1968) 3079–3084; *Anal. Abstr.*, 18 (1970) 1116—cellulose acetate.

11. ORGANIC ACIDS AND LIPIDS

11b. Lipids and their constituents

REZTSOVA, V. V.: (Determination of acetalphosphatides in human blood serum by agar gel electrophoresis). *Lab. Delo*, (1969) 27–29; *Anal. Abstr.*, 18 (1970) 2628—agar gel.

11c. Lipoproteins

LEWIS, L. A.: Screening for lipoprotein abnormalities: comparison of ultra-centrifugal, paper and thin-layer starch-gel electrophoresis techniques. *Lipids*, 4 (1969) 60–66; *Anal. Abstr.*, 18 (1970) 3344.

MOINUDDIH, M. AND TAYLOR, L.: Serum lipoproteins. Paper electrophoresis method without albumin in the buffer. *Lipids*, 4 (1969) 186–189; *Anal. Abstr.*, 19 (1970) 1612.

NARAYAN, K. A., MARY, G. E. AND FRIEDMAN, H. P.: Disc electrophoresis of bus-classes of human serum low-density lipoproteins. *Microchem. J.*, 14 (1969) 235–241; *Anal. Abstr.*, 19 (1970) 1613.

NOBLE, R. P., HATCH, F. T., MAZRIMAS, J. A., LINDGREN, F. T., JENSEN, L. C. AND ADAMSON G. L.: Comparison of lipoprotein analysis by agarose gel and paper electrophoresis with analytical ultra-centrifugation. *Lipids*, 4 (1969) 55–59; *Aanl. Abstr.*, 18 (1970) 3343.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BALESTRIERI, C., RAVA, R., DE PASCALE, C., ESPOSITO, R., DE SANTO, N. C. AND GIORDANO, C.: (Chromatographic method for the detection of methylurea). *Boll. Soc. Ital. Biol. Sper.*, 44 (1968) 2224–2226; *Anal. Abstr.*, 18 (1970) 2647—Whatman No. 3MM paper.

18. AMINO ACIDS

ESTAS, A.: Separation and analysis of amino acids. Two-dimensional high-voltage electrophoresis followed by (paper) chromatography. *Chromatogr. Electrophor. Symp. Int., 4th, 1966*, (Publ. 1968) 434–440; *C.A.*, 72 (1970) 87017u—paper.

FREIMUTH, U., NOETZOLD, H. AND FENK, L.: (Thin-layer electrophoretic detection of glutamic acid in foods). *Nahrung*, 13 (1969) 373–374; *Anal. Abstr.*, 19 (1970) 1720—silica gel–gypsum (9:1).

KRONMAN, J. H., TURESKY, S., CAMPBELL, C. O. AND KRASNOFF, M. M.: Micromethod for the determination of amino acids in human dental plaque. *J. Dent. Res.*, 48 (1969) 1315; *C.A.*, 72 (1970) 75463y.

LOKTIONOV, A. M.: (Separation of basic amino acids by a combination of paper electrophoresis

and paper chromatography). *Lab. Delo*, (1969) 14–15; *Anal. Abstr.*, 18 (1970) 2658—Whatman No. 1 paper.

MASSA, A., COCCHIERI, R. AND TENTORI, L.: (Thin-layer electrophoresis of dansyl derivatives of amino acids). *Ann. Ist. Sup. Sanita*, 5 (1969) 48–50; *Anal. Abstr.*, 18 (1970) 4247—silica gel.

TESTAFERATA, A., ZAMARCHI, E. AND LUTI, E.: (Combination of low-voltage electrophoresis and chromatography for the investigation of the plasma and urinary amino acids). *Riv. Clin. Pediat.*, 81 (1968) 631–635; *C.A.*, 72 (1970) 87003m—paper.

TESTAFERATA, A., ZAMARCHI, E. AND PIERRO, V.: (High-voltage electrophoresis in the identification of urinary amino acids). *Riv. Clin. Pediat.*, 81 (1968) 700–705; *C.A.*, 72 (1970) 87023t—paper.

TESTAFERATA, A., ZAMARCHI, E. AND SILENZI, M.: (Semi-quantitative determination of some amino acids by means of low-voltage electrophoresis). *Riv. Clin. Pediat.*, 81 (1968) 706–709; *C.A.*, 72 (1970) 87024u—paper.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

ROHRLICH, M. AND GALLERT, H.: (Isolation and identification of peptides from the peptic hydrolysate of wheat gliadin. I. Chromatographic and electrophoretic separation of peptic hydrolysates). *Z. Lebensm. Unters. Forsch.*, 137 (1968) 149–157; *Anal. Abstr.*, 17 (1969) 2941—Pherolan.

ROHRLICH, M. AND GALLERT, H.: (Isolation and identification of peptides from the peptic hydrolysate of wheat gliadin. II. Investigation of the isolated peptides). *Z. Lebensm. Unters. Forsch.*, 137 (1968) 223–231; *Anal. Abstr.*, 17 (1969) 2941—carrier not specified.

19b. Elucidation of structure of proteins

DERMINOT, J., TASDHOME, M. AND ALLARD, N.: (Determination of lysylalanine in wool hydrolysates). *Bull. Inst. Text. Fr.*, 24 (1970) 71–80; *C.A.*, 72 (1970) 133955y.

GÜRTLER, L. AND HORSZMANN, H. J.: Die Aminosäure-Sequenz vom Cytochrome C des Karpfens, *Cyprinus carpio*. *Eur. J. Biochem.*, 12 (1970) 48–57—polyacrylamide gel.

KOSTKA, V., MORÁVEK, L. AND ŠORM, F.: Amino acid sequence of C-terminal fragment of hog pepsin. *Eur. J. Biochem.*, 13 (1970) 447–454—paper.

20. PROTEINS (INCLUDING ENZYMES)

CHEN, CH. H. AND BUSHUK, W.: Nature of proteins in triticale (hybrid of wheat and rye) and its parental species. II. Gel filtration and disc electrophoresis results. *Can. J. Plant Sci.*, 50 (1970) 15–24; *C.A.*, 72 (1970) 86365u—polyacrylamide gel.

20a. Proteins of plant origin including bacteria

BAYLOR, M. B. AND ROSLANSKY, D. F.: Gel electrophoresis of the head proteins of T-even phage. *Virology*, 40 (1970) 251–259; *C.A.*, 72 (1970) 87302h—polyacrylamide gel.

GARCIA-FAURE, R., MERCK-LUENGO, J. G. AND GARCIA-OLMEDO, F.: Detection of flour or farina from *Triticum aestivum* in macaroni by starch gel electrophoresis of water-soluble proteins. *Cereal Chem.*, 46 (1969) 621–625—starch gel.

SCHWARTZ, J., SLOAN, J. AND LEE, Y. C.: Mannosidase, glucosidase and galactosidase in sweet almond emulsin. *Arch. Biochem. Biophys.*, 137 (1970) 122–127—electrofocusing.

TRAVERSO-CORI, A., TRAVERSO, S. AND REYES, H.: Different molecular forms of potato apyrase. *Arch. Biochem. Biophys.*, 137 (1970) 133–142—paper.

20b. Plasma proteins

ABDEL-SAMIE, Y. M., BEHELAK, Y. F., GHAZAL, A. R. M. AND HELMY, A. M.: Serum protein distribution by filter paper electrophoresis. I. Establishment of the method and standardization of normal Egyptians. *Alexandria Med. J.*, 15 (1969) 156–168; *C.A.*, 72 (1970) 87696q—paper.

EK, N.: Studies on electrophoresis on cellulose acetate membrane of bovine serum proteins in healthy animals. *Acta Vet. Scand.*, 10 (1969) 118–126; *Anal. Abstr.*, 18 (1970) 4256—microzone membrane.

LOSZA, A., KERESZTI, Z. AND BERENCSI, G.: (Electrophoretic determination of serum glycoproteins). *Ärztl. Lab.*, 14 (1970) 292–297; *Anal. Abstr.*, 18 (1970) 448.

LOSZA, A., KERESZTI, Z. AND BERENCSI, G.: (Possible differentiation of paraproteins by electrophoresis). *Z. Med. Labortech.*, 10 (1969) 205–207; *C.A.*, 72 (1970) 87027x—paper.

MAN, E. B. AND WHITEHEAD, JR., R. J.: Acrylamide-gel electrophoresis of serum proteins in infancy and pregnancy. *Clin. Chem.*, 14 (1968) 1002–1009; *Anal. Abstr.*, 18 (1970) 450—polyacrylamide gel.

MOGGI, C.: (Radial electrophoretic studies (with cellulose acetate gel) of serum proteins and gly-

coproteins in children with thalassemia major). *Riv. Clin. Pediat.*, 81 (1968) 728–731; *C.A.*, 72 (1970) 88056t—cellulose acetate.

MORIWAKI, K., TSUCHIYA, K. AND YOSIDA, T. H.: Genetic polymorphism in the serum transferrin of *Rattus rattus*. *Genetics*, 63 (1969) 193–199; *C.A.*, 72 (1970) 87841h—starch gel.

SEBASTIANI, A., ASSUMMA, M. AND BALESTRIERI, A.: (Standard electrophoretic diagram of the serum proteins in some tropical populations). *Arch. Ital. Sci. Med. Trop. Parassitol.*, 50 (1969) 107–118; *C.A.*, 72 (1970) 87839p—paper.

SOVETKIN, S. V., BELIKOVA, Z. A., BOROKOVA, E. V., SABIROV, M. S. AND NURULLAEV, L. D.: (X-Ray diffraction and electrophoretic study of protein components in donor and fibrinolysed blood). *Uzb. Biol. Zh.*, 13 (1969) 11–13; *C.A.*, 72 (1970) 76641s.

TKACHENKO, A. V.: (Method of over-all staining with a Procian dye of agar gel micro-electrophoretograms of proteins). *Vop. Med. Khim.*, 15 (1969) 100–104; *Anal. Abstr.*, 18 (1970) 3340—agar gel.

20c. Structural proteins

RATYCH, I. B.: (Electrophoretic study of soluble protein in cattle hides). *Fiziol. Biokhim. Sil's'kohospod. Tvarin*, 10 (1969) 47–50; *C.A.*, 72 (1970) 80366y—agar gel.

20e. Chromoproteins

STARODUB, N. F.: Disc electrophoretic fractionation of hemoglobin from irradiated animals. *Radiobiologiya*, 9 (1969) 909–911; *C.A.*, 72 (1970) 75371s—polyacrylamide gel.

20f. Varia, with special reference to non-identified and tissue proteins

ASCHAFFENBURG, R. AND DANCE, J. E.: Detection of cow's milk in goat's milk by gel electrophoresis *J. Dairy Res.*, 35 (1968) 383–384; *Anal. Abstr.*, 18 (1970) 552—polyacrylamide gel.

KURCZ, M., NAGY, I. AND BARANYAI, P.: (New biochemical method for the microdetermination of prolactin in the adenohypophysis of rats). *Probl. Endokrinol.*, 15 (1969) 63–69; *C.A.*, 72 (1970) 87022s—polyacrylamide gel.

LEARSON, R. J.: Collaborative study of a rapid electrophoretic method for fish species identification. II. Authentic flesh standards. *J. Ass. Offic. Anal. Chem.*, 53 (1970) 7–9; *C.A.*, 72 (1970) 77511m—cellulose acetate.

PAVLŮ, J.: (Fractionation of proteins from the cytoplasmic fraction of rat liver tissue using the method of electrofocusing and plate electrophoresis). *Čas. Lék. Čes.*, 109 (1970) 273–275; *C.A.*, 73 (1970) 41825y—isoelectric focusing.

TORRESANI, J., ROQUES, M. AND LISSITZKY, S.: Analysis of iodoproteins by electrophoresis on polyacrylamide gel. *Bull. Soc. Chim. Biol.*, 50 (1968) 1993–2002; *Anal. Abstr.*, 18 (1970) 1899—polyacrylamide gel.

20g. Enzymes: oxidoreductases

RUDDLE, F. H., CHAPMAN, V. M., CHEN, T. R. AND KLEBE, R. J.: Linkage between human lactate dehydrogenase A and B and peptidase B. *Nature*, 227 (1970) 251–257—polyacrylamide gel.

SPARKES, R. S., BALUDA, M. C. AND TOWNSEND, D. E.: Cellulose acetate electrophoresis of human glucose-6-phosphate dehydrogenase. *J. Lab. Clin. Med.*, 73 (1969) 531–534; *Anal. Abstr.*, 18 (1970) 4270—cellulose acetate.

20h. Enzymes: transferases

ANDERSEN, W. R., WILDNER, G. F. AND CRIDDLE, R. S.: Ribulose diphosphate carboxylase. III. Altered forms of ribulose diphosphate carboxylase from mutant tomato plants. *Arch. Biochem. Biophys.*, 137 (1970) 84–90—polyacrylamide gel.

HSU, D.-S. AND REEVES, R. E.: Improved purification of 1-phosphofructokinase from *Bacteroides symbiosus* and some properties of the enzyme. *Arch. Biochem. Biophys.*, 137 (1970) 59–64—polyacrylamide gel.

20i. Enzymes: hydrolases

DE LA LANDE, F. A. AND BOETTCHER, B.: Electrophoretic examination of human serum amylase isoenzymes. *Enzymologia*, 37 (1969) 335–342; *C.A.*, 72 (1970) 74892a—polyacrylamide gel.

DOLGO-SABUROV, V. B.: (Enzyme-electrophoretic separation of blood serum cholinesterase on agar gel). *Lab. Delo*, (1968) 740–741; *Anal. Abstr.*, 18 (1970) 1927—agar gel.

GALAEV, YU. V.: (Electrophoretic determination of L-asparaginase). *Prikl. Biokhim. Mikrobiol.*, 5 (1969) 207–210; *Anal. Abstr.*, 18 (1970) 2713.

KADOUM, A. M.: Simple starch-gel electrophoretic method to investigate esterase inhibition by common organic insecticides. *Bull. Environ. Contam. Toxicol.*, 4 (1969) 80–87; *Anal. Abstr.*, 19 (1970) 551—starch gel.

KAMINSKI, E. AND BUSHUK, W.: Wheat proteases. I. Separation and detection by starch-gel electrophoresis. *Cereal Chem.*, 46 (1969) 317–324; *Anal. Abstr.*, 19 (1970) 1723—starch gel.

RENNINGER, W. AND BIMBOESE, Ch.: (Adenosine deaminase–isoenzyme system). *Ärztl. Lab.*, 16 (1970) 139–143; *C.A.*, 73 (1970) 41922c.

20n. Enzymes: complex mixtures

MONTES DE OCA, F., MACY, M. L. AND SHANNON, J. E.: Isoenzyme characterization of animal cell cultures. *Proc. Soc. Exp. Biol. Med.*, 132 (1969) 462–469; *C.A.*, 72 (1970) 76461h—starch gel.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21b. Nucleic acids: RNA

ÖBERG, B. AND PHILIPSON, L.: Replication of poliovirus RNA studied by gel filtration and electrophoresis. *Eur. J. Biochem.*, 11 (1969) 305–315—polyacrylamide gel.

30. SYNTHETIC AND NATURAL DYES

TONET, N.: (Use of high-voltage electrophoresis as a supplementary technique for identification of water-soluble dyes). *Mitt. Geb. Lebensmittelunters. Hyg.*, 60 (1969) 201–205; *Anal. Abstr.*, 19 (1970) 1449.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

GARBER, C.: (Paper electrophoresis of some sulphonamides in therapeutic use. I). *Proanalisis*, 2 (1969) 16–21; *Anal. Abstr.*, 18 (1970) 4326—Whatman No. 1 paper.

33. INORGANIC SUBSTANCES

MEIER, H., ZIMMERHACKL, E., ALBRECHT, W., BOESCHE, D., HECKER, W., MENGE, P., RUCKDESCHEL, A., UNGER, E. AND ZEITLER, G.: Continuous electrophoretic separation of inorganic ions. I. Separations in the milligram to decigram regions. *Microchim. Acta*, (1970) 86–94; *C.A.*, 72 (1970) 128327r.

PARREIRA, H. C.: Electrophoresis of carbon black in liquids of low dielectric coefficient. Effect of the metallic element of the absorbate. *J. Electroanal. Chem. Interfacial Electrochem.*, 25 (1970) 69–78; *C.A.*, 72 (1970) 83323t—carrier-free electrophoresis.

SINGH, R. S., TRIPATHI, S. N., GJOSE, A. K. AND DEY, A. K.: Separation of platinum metals by paper electrophoresis. *Separ. Sci.*, 4 (1969) 171–179; *Anal. Abstr.*, 19 (1970) 1306.

TOMUS, M. AND TOTAN, M.: Separation of lanthanum from uranium by paper electrophoresis. *Stud. Univ. Babes-Bolyai, Ser. Chem.*, 13 (1968) 49–52; *Anal. Abstr.*, 17 (1969) 2641—Whatman No. 4 paper.

ZAITSEV, V. M., GUSEL'NIKOV, V. S. AND MAKHOMALKINA, S. M.: (Rapid chromatographic and electrophoretic methods for separating tellurium(IV) and tellurium(VI)). *Radiokhimiya*, 11 (1969) 352–353; *Anal. Abstr.*, 19 (1970) 1231.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

BARUEL, J. AND MARQUES, R. O.: Use of thin-layer electrophoresis in the radiochemical purity control of labelled compounds. *Radiochem. Radioanal. Lett.*, 2 (1969) 217–224; *C.A.*, 72 (1970) 83324u—cellulose, silica gel and cellulose acetate layers.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

GEIPERT, F., LEMBECK, F. AND SPROESSLER, B.: (Differentiation of substance P and physalaemin). *Naunyn-Schmiedebergs Arch. Pharmakol.*, 265 (1969) 225–232; *C.A.*, 72 (1970) 86407j—paper.

MEHRISHI, J. N.: Action of pronase and neuraminidase on the electrophoretic mobility of erythrocytes from normal cats and those with spontaneous tumors. *Vox Sang.*, 18 (1970) 27–33; *C.A.*, 72 (1970) 88232x.

CHROM. 5370

RESPONSE PREDICTION OF THE THERMAL CONDUCTIVITY DETECTOR WITH LIGHT CARRIER GASES

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SUMMARY

In gas chromatography the response of thermal conductivity detectors is dependent on the physical properties of the solute. This investigation determined that signal strengths represented as relative molar response factors can be calculated from the critical properties of both the chromatographic fraction and a carrier gas of low molecular weight. The approach suggested by LITTLEWOOD has been adopted and appropriately modified with the incorporation of a molecular weight term. The equation is applicable to both polar and non-polar compounds when helium or hydrogen are carrier gases. More significantly, the decrease in response with increased molecular symmetry for isomeric compounds is accurately predicted.

INTRODUCTION

Investigations[1–4] have confirmed that the signal strength arising from the presence of an eluted solute in a typical detector depends primarily on the nature of the solute. MESSNER *et al.*[2] reported that these relative molar response (RMR) factors were nearly a linear function of molecular weight within a homologous series and that the relative response of a branched hydrocarbon is less than the RMR of the normal (*n*-) isomer.

Several attempts have been made to calculate RMR factors theoretically. The path most frequently chosen utilizes an appropriate thermal conductivity mixture formula derived from the kinetic theory of gases. However, difficulties are encountered because thermal conductivities of the components in a gaseous binary mixture (eluted solute and carrier gas) are usually not additive. Furthermore the thermal conductivity of a mixture cannot be accurately determined in all cases by empirical equations.

HOFFMANN[5] employed the thermal conductivity relationship proposed by WASSILJEWA[6] and suggested that cell response is proportional to $(K_1 - K_{12})/K_{12}$. K_1 and K_{12} represent the thermal conductivity of the carrier gas and the mixture of solute and carrier gas, respectively. Similarly, LITTLEWOOD[7,8], LUY[9] and MECKE AND ZIRKER[10] proposed applications of the rigorous Chapman–Enskog theory[11,12]. Relevant to the present study is the extension of the Chapman–Enskog theory which was

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adopted by LITTLEWOOD[7]. For the case where the mole fraction of one component is small (X_2), LITTLEWOOD derived the following equation:

$$\frac{1}{K_1}\frac{dK_m}{dX_2} \cong -2.30\frac{\sigma_{12}^2}{\sigma_1^2} \quad (1)$$

where $\sigma_{12} = (\sigma_1 + \sigma_2)/2$. The molecular diameters of the carrier gas and solute are designated by σ_1 and σ_2, respectively. There were four assumptions made by LITTLEWOOD[8] in the derivation of this equation.

(1) X_2 must be much less than 1.

(2) The molecular weight of the solute (M_2) must be at least twenty times that of the carrier gas (M_1).

(3) Organic molecules may be regarded as rigid spheres.

(4) The validity of the Chapman–Enskog theory is unaffected by the internal structure of the solute.

LITTLEWOOD's equation illustrates how the thermal conductivity of the carrier gas is altered by the presence of solute molecules. As the carrier gas is responsible for the heat dissipation in the sensing filament, the solute vapor interferes with this process to the extent of their total cross-sectional area, namely, $X_2\sigma_{12}^2$. The resulting decrease in thermal conductivity is indicated by the minus sign.

LITTLEWOOD[8] stated that collision diameters for only a few compounds are available in the literature and also that the concept of a molecular diameter is not precise enough to make such values applicable in all circumstances. To alleviate these difficulties, he believed that it is feasible that the cross-sectional area of an organic molecule is approximately equal to the sum of the cross-sectional areas of its structural units. Analyzing the RMR data reported by ROSIE AND GROB[1], LITTLEWOOD found that the RMR factors of these compounds can be determined by addition of the RMR values assigned to each structural unit. However, this procedure is unsatisfactory for very symmetrical molecules, such as 3-ethylpentane.

Recently, NOVAK *et al.*[13] chose to predict RMR values through consideration of both conductive and convective heat effects. With hydrogen as the carrier gas, the calculated response factors agreed well with the experimental data, but theoretical predictions were inadequate with nitrogen as carrier gas due to the non-linear response of the detector and peak distortion for both the internal standard and the compound under consideration.

CONCEPT OF A MOLECULAR DIAMETER

The thermal conductivity of a vapor is dependent upon its molecular weight and distance of closest approach in addition to other molecular properties. The distance of closest approach may be defined by the σ term in the Lennard–Jones (12 — 6) intermolecular potential energy function,

$$V(r) = 4e_0\left[\left(\frac{\sigma}{r}\right)^{12} - \left(\frac{\sigma}{r}\right)^{6}\right] \quad (2)$$

in which e_0 is the maximum energy of attraction of two colliding molecules. The param-

eters σ and e_0 have been related to the viscosity and thermal conductivity of a gas near atmospheric pressure by the rigid Chapman–Enskog theory[12]:

$$N = \frac{0.002669\,(MT)^{1/2}}{\sigma^2\Omega^{(2,2)*}(T^*)} \qquad \text{[cP]} \tag{3}$$

$$K = \frac{1.989 \times 10^{-4}(T/M)^{1/2}}{\sigma^2\Omega^{(2,2)*}(T^*)} \qquad \text{[cal/(cm) (sec) (°K)]} \tag{4}$$

where M is the molecular weight of the gas and T is absolute temperature. The collision integral, $\Omega^{(2,2)*}(T^*)$, in which $T^* = kT/e_0$, is the resultant of a complex set of integrals after the potential function and temperature have been selected.

Since viscosity and thermal conductivity are a function of the Lennard–Jones parameters, these constants may be determined from the experimental measurements by choosing the set of σ and e_0 which fits the data most accurately. Complications

TABLE I

LENNARD–JONES PARAMETERS DETERMINED FROM VISCOSITY DATA

Compound	σ (Å)	e_0/k (°K)	*Reference*
n-Butane	4.687	531	14
	4.997	410	15
	5.339	310	16
	5.869	208	17
Benzene	5.349	412	14
	5.443	387	16
	5.628	412	17
	5.270	440	18

TABLE II

LENNARD–JONES PARAMETERS CALCULATED FROM VISCOSITY DATA FOR VARIOUS COMPOUNDS IN A HOMOLOGOUS SERIES

Compound	σ (Å)	e_0/k (°K)	*Reference*
Methane	3.808	140	17
Ethane	4.384	238	17
Propane	5.240	206	17
Butane	5.869	208	17
Pentane	6.099	269	17
Hexane	5.916	423	17
Heptane	—	—	no values reported
Octane	7.407	333	17
Nonane	8.302	266	17
Methanol	3.666	452	19
Ethanol	4.370	415	19
Dimethyl ether	4.264	412	19
Diethyl ether	5.539	351	19
Methyl acetate	5.054	417	19
Ethyl acetate	5.163	531	19

arise in this procedure as there are often multiple sets of σ–e_0 values which will reproduce the same viscosity data. Pairs of σ–e_0 values for *n*-butane and benzene are presented in Table I. In addition to the existence of several sets of parameters for a given compound, irregularities in reported values are common. The data in Table II show that the addition of a methylene group does not contribute a constant amount to either the collision diameter or the energy of interaction.

DETERMINATION OF COLLISION DIAMETERS

To eliminate irregular trends and multiple sets of σ–e_0 values, collision diameters have been calculated by the empirical expressions reported in the literature. In particular, TEE *et al.*[16] found that the following relations accurately reproduced experimental viscosity data for fourteen non-polar, non-associated substances:

$$\sigma = 2.36\ (T_c/P_c)^{1/3} \tag{5}$$

$$e_0/k = 0.774\ T_c \tag{6}$$

where T_c and P_c are the critical temperature (°K) and pressure (atm), respectively. Only the additional diameter expressions employed in the present investigation and the appropriate designation by which they will be subsequently referred to are presented in Table III.

TABLE III

COLLISION DIAMETER EXPRESSIONS

T_c = critical temperature (°K); P_c = critical pressure (atm); V_c = critical volume (cm³/gmole); W = acentric factor.

Designation	*Expression*	*Reference*
1	$\sigma = 2.3647\ (T_c/P_c)^{1/3}$	16
2	$\sigma = (2.3454 + 0.2972\ W)\ (T_c/P_c)^{1/3}$	16
3	$\sigma = 2.3442 \exp (0.1303\ W)\ (T_c/P_c)^{1/3}$	16
4	$\sigma = (0.8123 + 0.1678\ W)\ V_c^{1/3}$	16
5	$\sigma = 0.5894\ V_c^{0.4006}$	20
6	$\sigma = (2.3551 - 0.0874\ W)\ (T_c/P_c)^{1/3}$	16
7	$\sigma = 0.785\ V_c^{1/3}$	21
8	$\sigma = 0.561\ V_c^{5/12}$	17
9	$\sigma = 0.618\ V_c^{1/3} T_c^{1/18}$	17

The acentric factor, W, appearing in Table III was introduced by PITZER[22,23] as a correlating parameter to characterize the acentricity of a molecule and is mathematically written as:

$$W = -\log P_{v \cdot p_r} - 1.00/T_{r=0.7} \tag{7}$$

where T_r and $P_{v.p_r}$ are the reduced temperature and reduced vapor pressure, respectively. This technique of defining W results from the fact that it is sensitive to the value of the reduced vapor pressure near the normal boiling point. It has been found that for spherically symmetric species, such as argon, the value of $P_{v.p_r}$, at a reduced

TABLE IV

COLLISION DIAMETERS (Å) OF SELECTED COMPOUNDS

Compound	*Collision diameter expression*		
	1	*3*	*8*
n-Pentane	5.71	5.85	6.13
n-Hexane	6.07	6.25	6.58
n-Heptane	6.41	6.66	6.99
n-Octane	6.73	7.03	7.39
n-Nonane	7.04	7.39	7.74
2,2-Dimethylbutane	5.94	6.10	6.51
2-Methylpentane	6.02	6.20	6.57
3-Methylpentane	6.00	6.17	6.55
2-Methylhexane	6.36	6.59	7.00
3-Methylhexane	6.31	6.53	6.94
3-Ethylpentane	6.29	6.50	6.92
2,2,3-Trimethylbutane	6.17	6.33	6.77
Benzene	5.34	5.45	5.69
Toluene	5.73	5.89	6.17
o-Xylene	6.16	6.35	6.59
m-Xylene	6.17	6.38	6.64
p-Xylene	6.22	6.41	6.65
1,3,5-Trimethylbenzene	6.40	6.70	7.02
Diethyl ether	5.57	5.69	5.82
Acetone	5.23	5.40	5.22
Methanol	4.42	4.71	4.09
Ethanol	4.76	5.13	4.73
Helium	3.14	3.11	3.04
Hydrogen	3.25	3.22	3.19

temperature equal to 0.7, is approximately 0.1; thus, $W = 0$. More significantly, the acentric factor is a convenient measure of the difference between a given molecule and an inert gas.

These diameter expressions generate a consistent set of diameters on which response prediction can be based. Diameters calculated by expressions 1, 3, and 8 are illustrated in Table IV for a few selected compounds. By inspection of Table IV it can be seen that collision diameters increase linearly with molecular weight for compounds belonging to a homologous series. Also, the diameter of a branched compound is smaller than that of the corresponding normal isomer, *e.g.*, 2-methylpentane and hexane. Furthermore, a decrease in collision diameter is associated with increased molecular symmetry as a comparison of 2-methylhexane, 3-methylhexane, and 3-ethylpentane indicates.

Critical constants and acentric factors for many organic compounds have been tabulated[24]. For compounds whose molecular constants are unavailable, their critical temperature and volume were estimated by LYDERSEN's method[25], the critical pressure by RIEDEL's procedure[26] and the acentric factor by the technique proposed by EDMISTER[27].

FORMULATION OF AN RMR EXPRESSION

When helium and hydrogen are employed as carrier gases, response behavior for compounds of vastly different functionalities can be predicted very well by the following equation:

$$RMR_i = \left[\frac{\frac{\sigma_i + \sigma_1}{\sigma_1}}{\frac{\sigma_\phi + \sigma_1}{\sigma_1}}\right]^2 \left[\frac{M_i - M_1}{M_\phi - M_1}\right]^{1/4} \times 100 \qquad (8)$$

The subscripts i, 1, and ϕ refer to the solute under consideration, the carrier gas, and benzene (the internal standard), respectively. The first bracketed term in the expression has been proposed by LITTLEWOOD[7], whereas the second term was incorporated in the present study to explain the increase in response with a corresponding increase in molecular weight. The factor of 100 represents the response of benzene, arbitrarily assigned a value of 100 response units per mole. In the calculation of an RMR value, the collision diameters of the substances indicated in the above equation were computed from the same diameter expression.

EXPERIMENTAL

Although this investigation was not designed to be experimentally oriented, the RMR of selected compounds were measured with helium as a carrier gas. Hydrogen was not employed as a carrier gas with the thermal conductivity detector.

Apparatus

The injection port and column were located in a Wilkens aerograph Model 600-B. The detector oven connected externally to the column exit was a Research Specialties Model 1601-2 equipped with a proportioning temperature controller capable of maintaining a temperature to $\pm 0.1°$. The column exit and the thermal conductivity cell were connected by stainless steel tubing tightly wrapped in a Briskeat heating tape. The temperature of the heating tape was controlled by a Variac autotransformer and maintained at 100°. Two partitioning columns were used: (1) a 6 ft. × 1/8 in. O.D. stainless steel column containing 20 % dinonyl phthalate on 60–80 mesh Chromosorb P; (2) a 6 ft. × 1/4 in. O.D. aluminum tubing containing 5.5 % Carbowax 20M on Fluoropak 80.

A Gow-Mac Model 9677 thermal conductivity detector was operated at 100° and with a bridge current of 10 mA supplied by a Gow-Mac power unit Model 9999-D1616. The detector elements were matched glass bead thermistors having a resistance (cold) of 8000 Ω. The flow rate of helium was controlled at 37 ml/min by a rotameter (Matheson Company). Peak areas were measured by a Nester Faust Summatic 1501 digital electronic integrator and the chromatographic signals were observed on a Leeds and Northrup Speedomax H recorder with a 0–1 mV range and a chart speed of 30 in./h. Injected sample size was less than 1 μl. The selected compounds were obtained from Matheson, Coleman and Bell and their purity was greater than 99 mole %.

Each compound was mixed with a known weight of benzene or toluene from which the mole % of each component could be calculated. Each mixture was chromatographed a minimum of three times and two mixtures were prepared for each compound.

PREDICTED RMR DATA WITH HELIUM AS CARRIER GAS

The predicted response factors of 68 compounds have been calculated by eqn. 8 and are presented in Table V as a function of the particular collision diameter expression employed in the calculations. Included for comparison are the experimental RMR factors reported by MESSNER *et al.*[2]. The experimental RMR data in parentheses were determined in the present investigation. Several conclusions may be drawn from the inspection of Table V. The predicted RMR values reflect the fact that relative

TABLE V

PREDICTED RMR FACTORS WITH HELIUM AS CARRIER GAS

Compound	*RMR experimental*	*RMR predicted*								
		1[b]	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Pentane	105 (106)	107	107	107	107	108	106	106	108	105
Hexane	123 (122)	121	123	123	122	123	120	119	125	120
Heptane	143 (136)	135	139	139	137	139	134	132	141	134
Octane	160 (151)	149	155	155	153	155	148	145	157	148
Nonane	177 (164)	164	171	171	167	170	162	157	173	162
2,2-Dimethylbutane	116	118	119	119	119	122	117	118	123	118
2,3-Dimethylbutane	116	118	119	119	119	122	118	117	123	118
2-Methylpentane	120	120	121	122	122	123	119	119	124	119
3-Methylpentane	119	119	121	121	121	123	119	119	124	119
2,2-Dimethylpentane	133	130	132	132	132	135	130	129	137	130
2,3-Dimethylpentane	135	130	133	133	132	135	130	129	137	131
2,4-Dimethylpentane	129	132	135	135	135	138	132	131	140	133
2-Methylhexane	136	134	137	137	137	139	133	132	141	134
3-Methylhexane	133	132	135	135	135	138	132	131	139	133
3-Ethylpentane	131	132	134	135	134	137	131	130	139	132
2,2,3-Trimethylbutane	129	129	130	130	129	133	128	127	135	129
2,2,4-Trimethylpentane	147	144	147	147	148	154	144	144	157	147
Cyclopentane	97	97	97	97	97	97	97	97	97	97
Methylcyclopentane	115	111	112	112	112	114	111	111	114	112
Ethylcyclopentane	126	125	127	127	126	129	125	124	130	126
Cyclohexane	114 (110)	110	110	110	109	111	110	109	112	110
Methylcyclohexane	120	124	125	125	121	123	124	119	124	121
Ethylene	48	56	55	55	54	53	57	56	52	52
Propylene	63	73	72	72	72	71	73	73	70	70
Isobutylene	82	88	88	88	87	87	88	88	87	86
1-Butene	81	88	88	88	88	88	88	89	88	86
cis-2-Butene	87	89	90	90	89	87	89	88	87	86
trans-2-Butene	85	87	88	88	89	88	87	89	88	87
1,3-Butadiene	80	85	85	85	84	83	85	85	83	83
Benzene	100	100	100	100	100	100	100	100	100	100
Toluene[a]	116 (114)	114	115	115	115	116	114	113	116	115
o-Xylene[a]	130 (124)	130	132	132	129	130	129	126	132	129
m-Xylene[a]	131 (128)	130	133	133	131	132	130	127	133	129
p-Xylene[a]	131	132	134	134	130	132	131	127	133	130
Ethylbenzene[a]	129	127	129	130	130	131	126	126	133	129

(*continued on p. 276*)

TABLE V (*continued*)

Compound	*RMR experimental*	*RMR predicted*								
		1[b]	2	3	4	5	6	7	8	9
Propylbenzene[a]	145	143	147	147	145	148	142	140	150	144
Isopropylbenzene[a]	142	143	149	149	149	148	141	140	150	144
p-Ethyltoluene[a]	150	144	148	148	145	147	142	139	148	143
1,2,4-Trimethylbenzene[a]	150	143	147	147	146	147	141	139	148	143
1,3,5-Trimethylbenzene[a]	149 (143)	142	147	147	147	147	140	139	148	143
Ethyl acetate[a]	111 (108)	111	114	114	112	108	111	107	109	107
Isopropyl acetate[a]	121	121	124	125	124	123	120	120	124	120
Butyl acetate[a]	135	137	141	141	139	139	136	133	140	135
Isoamyl acetate[a]	145	149	153	154	150	153	148	144	155	148
Diethyl ether	110 (107)	104	104	104	101	101	104	101	101	100
Dipropyl ether[a]	131	129	133	134	134	133	128	127	135	129
Diisopropyl ether	130	129	132	132	131	132	128	126	133	127
Butyl ether[a]	160	157	165	165	164	163	155	151	166	156
Ethyl butyl ether[a]	130	130	134	134	134	133	128	127	135	129
Argon	44	51	49	49	49	47	51	52	45	45
Nitrogen	42	49	47	47	48	45	49	50	44	43
Oxygen	40	47	46	46	46	44	48	49	42	43
Carbon monoxide	42	49	48	48	48	46	50	51	45	44
Carbon dioxide	48	57	59	59	60	53	57	58	51	53
Methanol	55 (55)	62	65	65	62	53	61	57	52	55
Ethanol	72 (72)	75	80	80	80	70	74	73	69	71
Propanol	83 (86)	90	95	96	95	85	88	87	85	86
Isopropanol[a]	85 (85)	91	99	99	99	86	88	87	85	86
Butanol[a]	95 (99)	103	110	110	111	101	101	101	102	101
sec.-Butanol[a]	97	103	110	110	109	100	102	100	100	100
tert.-Butanol[a]	96 (96)	103	110	111	111	101	101	101	102	100
3-Methyl-1-butanol[a]	107	115	123	124	121	109	112	108	109	108
Acetone	86 (83)	90	91	91	87	83	90	85	83	84
Methyl ethyl ketone	98	103	105	105	102	99	102	99	99	99
Diethyl ketone	110	115	118	118	119	117	114	114	118	115
3,3-Dimethyl-2-butanone[a]	118	127	129	129	127	127	126	123	128	124
Methyl amyl ketone[a]	133	143	150	150	147	145	141	137	147	140
Methyl hexyl ketone[a]	147	156	165	166	162	159	154	149	162	154

[a] Estimation of critical constants.
[b] The numbers 1–9 refer to the collision diameter expressions as given in Table III.

response is indeed a linear function of molecular weight for compounds of a homologous series. In the case of the alkanes, slightly better agreement is obtained when the experimental factors of our study serve as the basis for comparison. The calculated RMR factors of branched compounds are less than the response factors of their corresponding normal isomer. An example of this effect is the isomeric pair 2,2-dimethylbutane and *n*-hexane. Moreover, eqn. 7 can also account for the decrease in response associated with increased molecular symmetry for isomers, *e.g.*, 2-methylhexane, 3-methylhexane, 3-ethylpentane, and 2,2,3-trimethylbutane.

Other trends in response are very adequately predicted as can be observed by a comparison of the computed and experimental factors of 1,3-butadiene with those of the isomeric butenes. In addition, surprisingly good agreement between the two sets of RMR values is achieved for the aromatics, acetates and ethers although their critical constants have been estimated. For the permanent gases studied, the calcu-

TABLE VI

PREDICTED RMR FACTORS WITH HYDROGEN AS CARRIER GAS

Compound	*Range of experimental factors*[a]		*RMR predicted*								
	Ref. 5	*Ref. 28*	*1*[b]	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Methane	41	—	44	42	42	43	41	44	45	40	41
Ethane	58	—	61	60	60	60	59	61	62	58	59
Propane	73	—	77	76	76	76	75	77	77	75	74
Butane	87	—	91	91	91	91	91	91	91	91	90
Pentane	104	102	106	107	107	106	107	106	105	107	104
Hexane	118	123	120	122	122	121	122	120	118	124	118
Heptane	133	132	134	138	138	136	138	133	131	140	132
Octane	150	152	148	153	154	151	153	147	143	156	145
Nonane	—	185	162	169	169	165	168	160	155	171	158
Benzene	100	100	100	100	100	100	100	100	100	100	100
Toluene	112	116	113	115	115	115	115	113	113	115	114
Cyclohexane	106	105	109	109	109	108	111	109	109	111	109
Diethyl ether	112	101	103	104	104	101	101	103	100	101	99
Acetone	86	89	90	91	91	87	83	89	85	83	84
Dichloromethane	—	85	91	91	91	91	89	91	92	88	90
Chloroform	—	99	104	104	104	107	107	104	107	106	107
Carbon tetrachloride	—	112	122	121	121	121	122	122	121	122	121
Carbon disulfide	—	84	81	80	80	81	80	81	83	79	82
Methanol	62	59	63	66	66	62	54	62	58	53	56
Ethanol	75	69	75	80	80	80	70	74	73	69	71
Propanol	—	—	89	95	95	95	85	88	87	85	86
Isopropanol	—	—	90	98	99	99	86	88	87	85	86
Butanol	—	—	103	109	110	110	101	101	100	101	101
Isobutanol	—	—	103	109	110	110	101	101	100	101	100
Pentanol	—	—	116	122	123	123	117	115	114	117	115
Ethyl acetate	—	—	111	113	114	111	108	110	107	108	106

[a] Indicates absence of experimental data.
[b] The numbers 1–9 refer to the collision diameter expressions as given in Table III.

lated RMR factors are slightly greater than the experimental ones. However, it is quite possible that these deviations may be attributed to techniques by which RMR values of gaseous substances are determined.

Conceding an error of 3% in the experimental measurement of a response factor, the overall agreement can be considered excellently, especially for those compounds whose molecular constants have been estimated. Essentially the accuracy of the predicted factors depends on the accuracy of the molecular constants and the validity of the exponent of the molecular weight term in eqn. 7. An exponent of 1/2 produces slightly better agreement for the permanent gases but a value of 1/4 is satisfactory for the overall molecular weight range considered.

PREDICTED RMR DATA WITH HYDROGEN AS CARRIER GAS

Although hydrogen is not ordinarily used as a carrier gas in conjunction with the thermal conductivity detector, a limited amount of RMR data has been reported[5,28] and is presented in Table VI together with the computed factors of twenty

compounds. The ability of a given collision diameter expression to accommodate both polar and non-polar substances is again demonstrated. In addition, the experimental and calculated RMR data are almost identical with those determined with helium as carrier gas. This agreement can be justified by considering the molecular weight and collision diameters of these gases. Since the molecular weight of both carrier gases is much less than that of most organic compounds, the magnitudes of the molecular weight term in the RMR equation are nearly identical for a particular solute with each carrier gas. Moreover, the collision diameters of helium and hydrogen are approximately equal, as can be seen in Table IV.

CONCLUSIONS

This investigation has determined that the concept of a collision diameter, which is specified in the Lennard–Jones potential function, may be successfully utilized in the calculation of response factors for a typical thermal conductivity detector. Because the diameter expressions are a function of molecular properties, the computed diameters provide a measure of the size of the solute vapor relative to the size of the carrier gas. However, these calculated diameters should not be confused with the concept of a "true" molecular diameter.

The independence of the computed RMR on molecular polarity can be explained by consideration of the environment of the chromatographic solute. In a gas chromatographic analysis, the mole fraction of eluted solute is much less than unity; thus, solute–solute interactions are minimized whereas solute–carrier gas interactions predominate. Consequently, the calculation of diameters for both non-polar and polar compounds by the same expression is justified because the appropriate molecular constants serve as a relative measurement of size. Furthermore, molecular interactions, such as hydrogen bonding and dipole–dipole effects, are essentially absent for polar substances under conventional gas chromatographic conditions.

With nitrogen as carrier gas, our RMR equation failed to predict the experimental data. Response prediction with this carrier gas will be the subject of a later communication.

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REFERENCES

1 D. M. ROSIE AND R. L. GROB, *Anal. Chem.*, 29 (1957) 1263.
2 A. E. MESSNER, D. M. ROSIE AND P. A. ARGABRIGHT, *Anal. Chem.*, 31 (1959) 230.
3 R. S. FISCHER, *Thermal Conductivity Cell Response*, Master of Science Thesis, University of Rhode Island, 1965.
4 L. A. HORROCKS, D. G. CORNWELL AND J. B. BROWN, *J. Lipid Res.*, 2 (1961) 92.
5 E. G. HOFFMANN, *Anal. Chem.*, 34 (1962) 1217.
6 A. WASSILJEWA, *Physik. Z.*, 5 (1904) 737.
7 A. B. LITTLEWOOD, *Nature*, 184 (1959) 1631.
8 A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New York, London, 1962, p. 306.

9 H. LUY, *Z. Anal. Chem.*, 194 (1963) 241.
10 R. MECKE AND K. ZIRKER, *J. Chromatogr.*, 7 (1962) 1.
11 S. CHAPMAN AND T. G. COWLING, *The Mathematical Theory of Non-Uniform Gases*, 2nd ed., Cambridge University Press, London, New York, 1952.
12 J. O. HIRSCHFELDER, C. F. CURTISS AND R. B. BIRD, *Molecular Theory of Gases and Liquids*, Wiley, New York, 1954, p. 534.
13 J NOVAK, S. WICAR AND J. JANÁK, *Coll. Czech. Chem. Commun.*, 33 (1968) 3642.
14 R. A. SVEHLA, *NASA Tech. Rept.*, R-132, Lewis Research Center, Cleveland, Ohio, 1962.
15 T. TITANI, *Bull. Chem. Soc. Jap.*, 5 (1930) 98.
16 L. S. TEE, S. GOTOH AND W. E. STEWART, *Ind. Eng. Chem. Fundam.*, 5 (1966) 356.
17 L W. FLYNN AND G. THODOS, *Amer. Inst. Chem. Eng. J.*, 8 (1962) 362.
18 LANDOLT–BORNSTEIN, *Physikalisch-Chemische Tabellen*, 5th ed., Springer, Berlin, 1923–1936.
19 E. A. MASON AND L. MONCHICK, *J. Chem. Phys.*, 35 (1961) 1676.
20 N. H. CHEN AND D. F. OTHMER, *J. Chem. Eng. Data*, 7 (1962) 37.
21 L. I. STIEL AND G. THODOS, *J. Chem. Eng. Data*, 1 (1962) 234.
22 K. S. PITZER, *J. Amer. Chem. Soc.*, 77 (1955) 3427.
23 K. S. PITZER, D. Z. LIPPMANN, R. F. CARL, C. M. HUGGINS AND D. E. PETERSON, *J. Amer. Chem. Soc.*, 77 (1955) 3433.
24 R. C. REID AND T. K. SHERWOOD, *The Properties of Gases and Liquids*, 2nd ed., McGraw-Hill, New York, 1966, pp. 571–584.
25 A. L. LYDERSEN, *Coll. Eng., Univ. Wis. Eng., Exp. Sta. Rept.*, 3, Madison, Wisc., April, 1955.
26 L. RIEDEL, *Z. Elektrochem.*, 53 (1949) 222.
27 W. C. EDMISTER, *Petrol. Refiner*, 37 (1958) 173.
28 N. HARA, S. YAMANO, Y. KUMAGAYA, K. IKELBE AND K. NAKAYAMA, *Kogyo Kagaku Zasshi*, 66 (1963) 1801.

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GAS–LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL DISACCHARIDES

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SUMMARY

The relative retention times of twenty-three trimethylsilyl disaccharides on three liquid phases varying in polarity, *viz.* OV-1, OV-17 and OV-25, are reported. Comparison of these values makes it clear that they are systematically influenced by each of the structural elements of the disaccharide.

INTRODUCTION

Gas–liquid chromatography (GLC) has become an important tool in the analysis of carbohydrates since the introduction of the trimethylsilyl (TMS) group as a protecting group by SWEELEY *et al.*[1]. A large amount of data are available on the GLC of TMS monosaccharides. The application of this technique to TMS ethers of disaccharides is mainly restricted to a few common representatives of this series[1–6], although PERCIVAL[7] investigated some less common ones.

In the course of our studies on the structure determination of carbohydrate-containing polymers we needed a method for the separation and identification of rather complex mixtures of oligosaccharides. With this in mind the GLC of TMS ethers of 23 disaccharides was studied. In this series of model compounds the possible variations in the position of the glycosidic bond are considered. Three liquid phases were tested for their suitability as column coatings *viz.* OV-1 (non-polar), OV-17 (medium-polar) and OV-25 (polar).

MATERIALS AND METHODS

Disaccharides

α,α-D-Trehalose dihydrate, β-D(+)-maltose monohydrate, β-D(+)-cellobiose, α-D(+)-lactose monohydrate, isomaltose, β-gentiobiose, α-D-melibiose monohydrate, D(+)-sucrose and D-lactulose were purchased from J. T. Baker Chemicals N.V.; turanose was purchased from Pierce Chemicals Company and palatinose from EGA-Chemie K.G. The following compounds were gifts: β,β-trehalose, laminaribiose, mannobiose, maniocose, 6-O-α-D-mannopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-galactose, 3-O-β-D-galactopyranosyl-D-arabinose, 2-O-β-D-glucopyranosyl-L-arabinose, α-kojibiose octaacetate, α-sophorose monohydrate, neolactose and prime-

TABLE I

R_s VALUES AND PEAK AREA RATIOS OF THE TMS-ETHERS OF DISACCHARIDES

Relative peak areas of the anomers are given in parentheses.

	Carbohydrate		*3% OV-1*	*3% OV-17*	*3% OV-25*
I	α-D-Glucopyranosyl-(1 → 1)-α-D-glucopyranoside	(α,α-trehalose)	1.34	1.38	1.31
II	β-D-Glucopyranosyl-(1 → 1)-β-D-glucopyranoside	(β,β-trehalose)	1.77	1.90	1.70
III	α-D-Glucopyranosyl-(1 → 2)-D-glucose	(kojibiose)	1.38 (10) 1.69 (7)	1.40 (10) 1.82 (7)	1.31 (10) 1.65 (7)
IV	β-D-Glucopyranosyl-(1 → 2)-D-glucose	(sophorose)	1.59 (3.5) 1.85 (10)	1.66 (3.5) 1.99 (10)	1.57 (3.5) 1.82 (10)
IV*	β-D-Glucopyranosyl-(1 → 2)-α-D-glucose		1.85	1.99	1.82
V	β-D-Glucopyranosyl-(1 → 3)-D-glucose	(laminaribiose)	1.56 (8) 1.76 (10)	1.64 (8) 1.80 (10)	1.53 (8) 1.62 (10)
VI	α-D-Glucopyranosyl-(1 → 4)-D-glucopyranose	(maltose)	1.12 (5) 1.30 (10)	1.19 (5) 1.33 (10)	1.18 (5) 1.26 (10)
VI*	α-D-Glucopyranosyl-(1 → 4)-β-D-glucopyranose		1.30	1.33	1.26
VII	β-D-Glucopyranosyl-(1 → 4)-D-glucopyranose	(cellobiose)	1.15 (7) 1.67 (10)	1.22 (7) 1.70 (10)	1.21 (7) 1.57 (10)
VII*	β-D-Glucopyranosyl-(1 → 4)-β-D-glucopyranose		1.67	1.70	1.57
VIII	β-D-Galactopyranosyl-(1 → 4)-D-glucopyranose	(lactose)	1.00 (7) 1.50 (10)	1.09 (7) 1.54 (10)	1.10 (7) 1.44 (10)
VIII*	β-D-Galactopyranosyl-(1 → 4)-α-D-glucopyranose		1.00	1.09	1.10
IX	β-D-Galactopyranosyl-(1 → 4)-D-altropyranose	(neolactose)	0.85	0.81 (4) 0.87 (10)	0.86 (4) 0.88 (10)

X	β-D-Mannopyranosyl-(1 → 4)-D-mannopyranose	(mannobiose)	1.21	(10)	1.18	(10)	1.30	(10)
			1.59	(4)	1.52	(4)	1.40	(4)
XI	α-D-Glucopyranosyl-(1 → 5)-D-glucofuranose	(maniocose)	2.01	(4.5)	2.40	(4.5)	2.15	(4.5)
			2.49	(10)	2.80	(10)	2.42	(10)
XII	α-D-Glucopyranosyl-(1 → 6)-D-glucose	(isomaltose)	2.02	(3)	2.37	(3)	2.14	(3)
			2.48	(10)	2.77	(10)	2.42	(10)
XIII	β-D-Glucopyranosyl-(1 → 6)-D-glucose	(gentiobiose)	2.02	(4)	2.36	(4)	2.14	(4)
			2.50	(10)	2.76	(10)	2.40	(10)
XIV	α-D-Galactopyranosyl-(1 → 6)-D-glucose	(melibiose)	2.02	(5)	2.16	(5)	1.98	(5)
			2.26	(10)	2.33	(10)	2.08	(10)
XIV*	α-D-Galactopyranosyl-(1 → 6)-α-D-glucose		2.02		2.16		1.98	
XV	α-D-Mannopyranosyl-(1 → 6)-D-glucose		1.55	(9)	1.70	(9)	1.59	(9)
			1.98	(10)	2.08	(10)	1.85	(10)
XVI	β-D-Galactopyranosyl-(1 → 6)-D-galactose		2.26		2.38		2.08	
XVII	β-D-Glucopyranosyl-(1 → 2)-L-arabinose		0.76	(8)	0.95		0.92	
			0.83	(10)				
XVIII	β-D-Galactopyranosyl-(1 → 3)-D-arabinose		0.63	(10)	0.73	(10)	0.75	(10)
			0.74	(6)	0.90	(6)	0.90	(9)
			0.80	(5)	1.03	(6)		
XIX	β-D-Xylopyranosyl-(1 → 6)-D-glucose	(primeverose)	1.49		1.92	(7)	1.68	(10)
					1.96	(10)	1.78	(7)
XX	α-D-Glucopyranosyl-(1 → 2)-β-D-fructofuranoside	(sucrose)	1.00		1.00		1.00	
XXI	α-D-Glucopyranosyl-(1 → 3)-D-fructose	(turanose)	1.28		1.28	(10)	1.21	(10)
					1.56	(4)	1.47	(4)
XXII	β-D-Galactopyranosyl-(1 → 4)-D-fructose	(lactulose)	1.00		0.94		0.97	
XXIII	α-D-Glucopyranosyl-(1 → 6)-D-fructofuranose	(palatinose)	1.25	(1)	1.39	(2)	1.34	(2)
			1.35	(2)	1.48	(10)	1.40	(10)
			1.43	(10)	1.79	(1)	1.70	(1)

verose. Sugars were anomerized in water for 48 h at room temperature, and subsequently lyophilized.

Preparation of TMS derivatives

The disaccharides were converted to the TMS derivatives by means of hexamethyldisilazane (Koch Light Ltd.) and trimethylchlorosilane (Schuchardt) in pyridine as described earlier[8].

Gas–liquid chromatography

An F and M gas chromatograph Model 700 equipped with a dual flame ionization detector and coiled stainless steel columns (2.70 m × 3.2 mm O.D.) was used. The packing materials were: 3 % OV-1, 3 % OV-17 and 3 % OV-25 on Chromosorb W (H.P.), 80–100 mesh, and were obtained from Pierce Chemicals Company. The temperature conditions were the following: injection port 270°, detector 310°, column oven 228°. The gas flow rates for H_2 and air were 45 ml/min and 375 ml/min, respectively. The gas flow rate of the carrier gas N_2 was 26 ml/min on 3 % OV-1, 18 ml/min on 3 % OV-17 and 5 ml/min on 3 % OV-25.

RESULTS AND DISCUSSION

The R_s values and peak area ratios for the TMS-derivatives of disaccharides I–XXIII on the three stationary phases are presented in Table I. TMS-sucrose was used as an internal standard ($R_s = 1.00$); the retention times of this compound were 14.6, 12.1 and 13.1 min on OV-1, OV-17 and OV-25, respectively. In the cases of α-D-G*p*-(1→2)-D-G (III), α-D-Man*p*-(1→6)-D-G (XV) and β-D-Gal*p*-(1→6)-D-Gal (XVI) in addition to the peaks mentioned in Table I, there are some small peaks which probably represent the furanose forms. The gas chromatograms of α-D-G*p*-(1→5)-D-G*f* (XI), α-D-G*p*-(1→6)-D-G (XII) and β-D-G*p*-(1→6)-D-G (XIII) show a great similarity. There are a few minor peaks present besides the main peaks which were attributed to the furanose forms of XI and the pyranose forms of XII and XIII.

Stationary phases

The three liquid phases OV-1 (non-polar), OV-17 (medium-polar) and OV-25 (polar) differ in the ratio of phenyl to methyl groups in the silicone oil. In general OV-17 gives the best separation, although, incidentally, OV-1 has definite advantages, for *e.g.* in the resolution of the following pairs of compounds: α-D-G*p*-(1→4)-D-G*p* (VI) and β-D-Man*p*-(1→4)-D-Man*p* (X); β-D-G*p*-(1→2)-L-Ara (XVII) and β-D-Gal*p*-(1→4)-D-Fru (XXII); and α-D-Man*p*-(1→6)-D-G (XV) and β-D-G*p*-(1→4)-D-G*p* (VII). OV-1 and OV-17 are nearly equivalent in their ability to separate anomeric forms, and can be used in preference to OV-25, except in the case of β-D-Xyl*p*-(1→6)-D-G (XIX). This aspect of fractionating anomeric forms is important because reducing sugars, obtained as breakdown products from oligo- or polymers, will mostly consist of mixtures of anomers. The occurrence of different forms may be an advantage as the combination of R_s and peak area data can be used for the identification of the disaccharides.

The suitability of OV-17 for the separation of TMS disaccharides is illustrated in Fig. 1. In complex mixtures the R_s values of the components remained identical

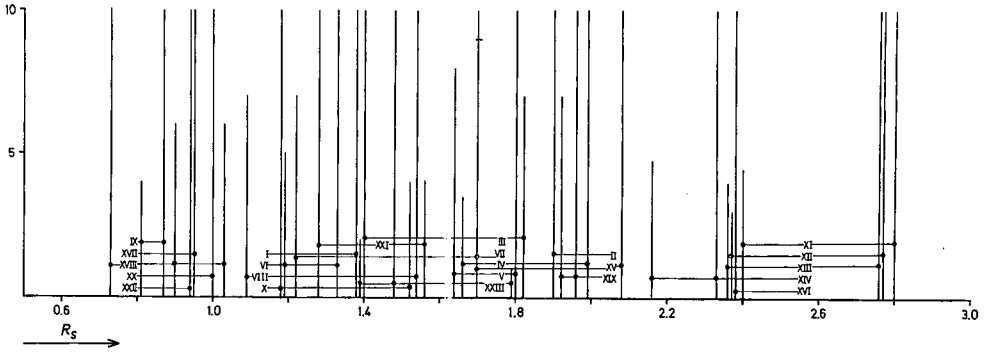

Fig. 1. The data compiled in Table I are graphically presented in this figure. The R_s values are given on the abcissa and the ratio of the peak areas of the anomers of each disaccharide on the ordinate. At $R_s = 1.70$ two peaks coincide.

to those listed in Table I. The resolution of α-D-G*p*-(1→5)-D-G*f* (XI), α-D-G*p*-(1→6)-D-G (XII) and β-D-G*p*-(1→6)-D-G (XIII) could not be achieved on any of the stationary phases.

Comparison of R_s values

Table I shows that for the (1→4) and (1→6) aldohexosyl-aldohexoses: α-D-G*p*-(1→4)-D-G*p* (VI); β-D-G*p*-(1→4)-D-G*p* (VII); β-D-Gal*p*-(1→4)-D-G*p* (VIII) and α-D-Gal*p*-(1→6)-D-G (XIV), which all contain D-glucose at the reducing end, the β-anomer has a longer retention time than the α-anomer. These results are analogous to the observations of SWEELEY, who found that the monosaccharide form with an equatorial hydroxyl group on C_1 has the longest retention time, provided that the stable conformation is the chair form 1C or 1C. However, β-D-G*p*-(1→2)-D-G (IV) shows the reverse sequence of anomers This anomalous behaviour may be a consequence of the bulky substituent at C_2 of the reducing D-glucose unit. It would be worthwhile investigating whether this feature is specific for (1→2)-aldohexosyl-aldohexoses. The reversal of the R_s values of the primeverose anomers (XIX) going from OV-17 to OV-25 indicates, that when the R_s values of the anomers are very close together, the stationary phase may influence the sequence of the peaks.

Comparison of the R_s values of TMS disaccharides, differing only in the con-

TABLE II

COMPARISON OF THE R_s VALUES OF DISACCHARIDES DIFFERING IN THE CONFIGURATION OF THE GLYCOSIDIC BOND

	Carbohydrate	*R_s on OV-1*	*R_s on OV-17*	*R_s on OV-25*
I	α-D-G*p*-(1→1)-α-D-G*p*	1.34	1.38	1.31
II	β-D-G*p*-(1→1)-β-D-G*p*	1.77	1.90	1.70
III	α-D-G*p*-(1→2)-D-G	1.38 and 1.69	1.40 and 1.82	1.31 and 1.65
IV	β-D-G*p*-(1→2)-D-G	1.59 and 1.85	1.66 and 1.99	1.57 and 1.82
VI	α-D-G*p*-(1→4)-D-G*p*	1.12 and 1.30	1.19 and 1.33	1.18 and 1.26
VII	β-D-G*p*-(1→4)-D-G*p*	1.15 and 1.67	1.22 and 1.70	1.21 and 1.57

TABLE III

EFFECT OF CHANGING THE ALDOHEXOSE UNIT ON THE R_S VALUE

	Carbohydrate	R_S *on OV-17*
VII	β-D-G*p*-(1 → 4)-D-G*p*	1.22 and 1.70
VIII	β-D-Gal*p*-(1 → 4)-D-G*p*	1.09 and 1.54
	β-D-G*p*	1.68[a]
	β-D-Gal*p*	1.08[a]
VIII	β-D-Gal*p*-(1 → 4)-D-G*p*	1.09 and 1.54
IX	β-D-Gal*p*-(1 → 4)-D-Alt*p*	0.81 and 0.87
	D-G*p*	(1.00 and 1.57)[b]
	D-Alt*p*	(0.65 and 0.68)[b]
XII	α-D-G*p*-(1 → 6)-D-G	2.37 and 2.77
XIV	α-D-Gal*p*-(1 → 6)-D-G	2.16 and 2.33
XV	α-D-Man*p*-(1 → 6)-D-G	1.70 and 2.08
	α-D-G*p*	1.05[a]
	α-D-Gal*p*	0.87[a]
	α-D-Man*p*	0.63[a]

[a] TMS-sorbitol is taken as an internal standard at 150°.
[b] Measured by SWEELEY *et al.*[1] on SE-52 at 140°, relative to α-D-G*p*.

figuration of the glycosidic bond (Table II) shows, that in general the components with the β-configuration have the longest retention times.

The influence of the constituent monosaccharides on the R_S values of the disaccharides can be demonstrated by comparison of disaccharides which differ in one monosaccharide (Table III). Replacement of an aldohexose unit X in a disaccharide by a stereoisomer Y results in a shift of the R_S value of the TMS disaccharide to lower values, in the case where the R_S value of Y is lower than that of X.

The changes in the R_S values of the disaccharides, observed so far, are restricted to components differing in one structural aspect only.

CONCLUSIONS

The optimal separation of TMS disaccharides is obtained on 3% OV-17 as stationary phase. Nevertheless it seems advisable to use at least two liquid phases with different polarity for the analysis of unknown mixtures.

For the identification of reducing sugars by GLC it may be advantageous to analyse equilibrium mixtures of anomeric forms. The combination of R_S values and peak areas gives more characteristic information about such a component than the single R_S value obtained after reduction to the corresponding alditol. However, for the preparative separation of disaccharides via GLC, reduction to alditols may be preferred as this conversion greatly diminishes the number of components in the mixture.

Comparison of R_S values of disaccharides which differ in one structural element, *viz.* the configuration of the anomeric C atom of the reducing unit, the configuration of the glycosidic bond, or the constituent monosaccharides, shows that at least qualitatively similar changes occur such as are known for monosaccharides.

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REFERENCES

1 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
2 A. E. PIERCE, *Silylation of Organic Compounds 1968–1969*, Pierce Chem. Co., Rockford, Ill.
3 E. G. HEDGLEY AND W. G. OVEREND, *Chem. Ind. (London)*, (1960) 378.
4 P. K. DAVISON AND R. YOUNG, *J. Chromatogr.*, 41 (1969) 12.
5 M. KIMURA, M. TOHMA, Y. OKAZAWA AND N. MURAI, *J. Chromatogr.*, 41 (1969) 110.
6 T. BHATTI, R. E. CHAMBERS AND J. R. CLAMP, *Biochim. Biophys. Acta*, 222 (1970) 339.
7 E. PERCIVAL, *Carbohyd. Res.*, 4 (1967) 441.
8 J. P. KAMERLING, J. F. G. VLIEGENTHART, J. VINK AND J. J. DE RIDDER, *Tetrahedron*, in press.

CHROM. 5332

GAS–LIQUID CHROMATOGRAPHY OF N-ACETYL-*p*-AMINOPHENOL (PARACETAMOL) IN PLASMA AND URINE

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SUMMARY

A procedure is described for determining unconjugated paracetamol in plasma and urine. The method, based on gas chromatography of the unchanged drug, is more rapid than a spectrophotometric assay and has a limit of detection of 2 μg/ml.

Plasma and urine levels are reported after therapeutic and overdose ingestion.

INTRODUCTION

The methods already described[1–3] for the determination of paracetamol in body fluids normally require hydrolysis of the drug to *p*-aminophenol, followed by a coupling reaction to form an azo dye which is then estimated spectrophotometrically. The reaction time for the hydrolysis in all of these procedures is usually about 1 h, so that when extraction and colour development are also included the analysis becomes lengthy and time consuming. This is a disadvantage for the clinical biochemist or toxicologist investigating cases of therapeutic and overdose ingestion.

ROUTH *et al.*[1] have recently compared a hydrolysis assay of this type with an assay employing the combination of the free radical dipicrylhydrazyl with paracetamol, and also with differential absorbance measurements of paracetamol at 266 nm. The dipicrylhydrazyl reaction with paracetamol also requires 1 h for its completion and was considered by the authors to be the least accurate of the techniques. The differential absorbance method in our hands gave higher blanks than those obtained by the authors and, more important, the molar extinction of paracetamol was not large enough for the measurement of some therapeutic samples.

A method has been developed therefore, using gas chromatography (GC) which is more rapid and sensitive than the procedures previously reported.

MATERIALS AND METHODS

Paracetamol was given to laboratory staff in the form of two 500 mg BP tablets per person or two Panasorb tablets (Sterling: Winthrop, Research and Development Division, Newcastle-upon-Tyne).

All other chemicals were obtained from Hopkin & Williams Ltd., Chadwell

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Heath, Essex. Diethyl ether (analar grade) was redistilled and the ammonium sulphate purified by washing with ether.

Diphenyl phthalate was used as the internal standard for the GC measurements. Two separate methanolic solutions were made up to contain: (i) 0.4 mg per 100 ml and (ii) 4 mg per 100 ml, respectively; the former being added when measuring therapeutic doses in the range 0.2–2 mg of paracetamol per 100 ml and the latter for overdoses in the range 2–20 mg of paracetamol per 100 ml.

Extraction of paracetamol from plasma

1 ml of plasma, derived from venous blood, was saturated with 3 g of solid ammonium sulphate and extracted twice with 15 ml of ether. The sample was centrifuged after each extraction and the ether layers pooled. 1 ml of the internal standard solution (i) or (ii) was added to the organic layer. The extract was transferred in small portions to a 10 ml conical tube (BC/C14T Quickfit & Quartz, Stone, Staffordshire) and evaporated to dryness under a stream of air. The residue was then carefully reconstituted in 0.1 ml of ether and 5 μl injected into the gas chromatograph.

Extraction of paracetamol from urine

Interfering compounds in the urine can be removed by the use of a Florisil column. A BTL chromatographic column, Type 2A, I.D. 1 cm (Baird & Tatlock, Chadwell Heath, Essex) was filled to a height of 8 cm with a slurry of Florisil which was then washed with 10 ml of distilled water and the excess allowed to drain. 30 ml of urine were then passed through this column, the first 10 ml were rejected and the remainder was collected. 1–5 ml of eluate was then saturated with solid ammonium sulphate and extracted twice with 15 ml of ether. Subsequent analysis was carried out by adding 1 ml of the internal standard solution and proceeding as described for plasma.

Gas–liquid chromatography

A Pye 104 gas–liquid chromatograph, equipped with a flame ionisation detector, was used for the analysis. The column was a 7 ft. glass coil previously silanised for 24 h with 5 % dimethyldichlorosilane in benzene, and packed with 2 % FFAP on Aeropak 30, mesh size 70–80 (Varian Aerograph, Fife, Great Britain). This was conditioned at 100° for 48 h without the carrier gas flowing. A further 48-h conditioning at 250° was made with nitrogen flowing at 60 ml/min. The instrument settings were: injection temperature, 280°; column temperature, 240°; detector temperature, 280°; gas flow rates: air, 400 ml/min; hydrogen, 45 ml/min; nitrogen, 60 ml/min; sensitivity 2×10^{-10} A.

A gradual deterioration of the column response and sensitivity occurred after three months continual use. Reconditioning of the column was necessary when this happened.

Measurements

Stock solutions were prepared so as to contain 20 mg of diphenyl phthalate in 100 ml of ether and 100 mg of paracetamol in 100 ml of ethanol–ether (5:95). These were diluted with ether so as to prepare a range of standard solutions containing 40 μg/ml of diphenyl phthalate and from 20–200 μg/ml of paracetamol, at intervals of 20 μg/ml. To construct the calibration curve, 2–5 μl of each of these samples were injected

on to the column. The retention times of paracetamol and diphenyl phthalate were 6.5 min and 11 min, respectively. The ratio of the peak heights of paracetamol to diphenyl phthalate was linear with respect to the concentration of paracetamol (Fig. 1). This line did not pass exactly through the origin, probably due to the slight tailing of the paracetamol peak. However, the calculation of paracetamol concentration from peak height ratio was used for the sake of simplicity, rather than the more precise peak area ratio which passed through the origin.

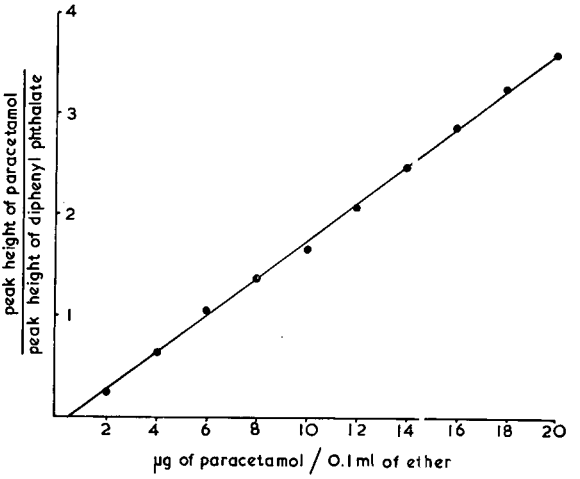

Fig. 1. The standard calibration graph of the ratio of the peak heights of paracetamol and diphenyl phthalate against the number of μg of paracetamol in the extract.

The concentration of the samples was calculated from this calibration curve by measuring the ratio of the peak heights of paracetamol to diphenyl phthalate in the sample and adjusting the figure obtained to account for the amount of internal standard employed. Thus no adjustment was necessary if the 0.4 mg/100 ml diphenyl phthalate solution was used. However, if the larger 4 mg/100 ml diphenyl phthalate solution was employed, the result must be multiplied by a factor of 10.

Interferences

There is the possibility that this assay may be applied to samples where phenacetin has been ingested and, therefore, its behaviour was investigated on the FFAP column. Phenacetin eluted after 1.5 min, while *p*-aminophenol (another metabolite of phenacetin that might be extracted) did not elute. This was expected since the hydroxy group renders it more polar than paracetamol.

Common drugs manufactured in tablet form in combination with paracetamol are aspirin, codeine and orphenadrine. Orphenadrine did not chromatograph, aspirin appeared in the solvent peak and codeine had a retention time of 7.5 min.

RESULTS

This method has been used for the analysis of therapeutic and overdose samples. Tables I and II show the plasma levels obtained when six normal adults had taken 1 g

of paracetamol BP or the equivalent amount of paracetamol in the form of a Panasorb (a mixture of paracetamol and sorbitol in the proportion 5:1). Free paracetamol plasma levels were generally lower than those obtained by spectrophotometric methods. However, GWILT *et al.*[6] have demonstrated that the absorption characteristics of paracetamol tablets differ with the source of the tablet, thus valid comparisons between our findings and those of other authors cannot be made. The difference between the plasma levels of paracetamol BP and Panasorb in the two tables shows that, usually, Panasorb is more rapidly absorbed to give higher levels than paracetamol B.P. Individual variations in the maximum plasma levels attained was quite

TABLE I

PLASMA PARACETAMOL LEVELS IN μg/ml

Volunteer				*Hours after ingestion of 1 g paracetamol B.P.*								
	Sex	*Age (yr)*	*Weight (kg)*	¼	½	1	1½	2	3	4	5	6
J.G.	M	34	73	—	7.1	10.1	9.2	8.2	4.3	3.2	1.7	0.9
D.B.	M	25	68	—	6.4	7.8	6.1	4.7	2.9	1.3	0.4	0.4
G.W.	M	24	73	—	0.4	0.3	4.2	5.7	3.9	3.1	2.9	1.9
L.C.	F	21	51	0	0	0	0.3	8.0	9.9	2.9	—	0.9
F.H.	F	22	70	0	3.4	13.3	9.2	7.0	4.1	2.7	—	0.7
B.H.	F	19	57	0	0	0	2.1	6.2	2.2	0.4	—	0

TABLE II

PLASMA PARACETAMOL LEVELS IN μg/ml

Volunteer				*Hours after ingestion of 1 g paracetamol in the form of Panasorb*								
	Sex	*Age (yr)*	*Weight (kg)*	¼	½	1	1½	2	3	4	5	6
J.G.	M	34	73	4.2	5.0	5.1	4.2	3.2	1.7	0.7	—	0
D.B.	M	25	68	8.9	14.8	10.3	8.4	6.3	3.1	2.1	—	0
G.W.	M	24	73	1.8	5.5	3.9	2.9	2.4	0.5	0.4	—	0
L.C.	F	21	51	3.6	4.1	6.8	8.5	14.3	11.1	5.4	—	1.6
F.H.	F	22	70	1.2	11.9	11.9	8.9	5.7	4.7	2.7	—	0.8
B.H.	F	19	57	0	0.8	3.2	7.3	16.8	6.4	6.7	—	3.0

TABLE III

TOTAL FREE PARACETAMOL VOIDED IN 24 h AFTER INGESTION OF PARACETAMOL AS PERCENTAGE DOSE

Volunteer		*Paracetamol*	*Panasorb*
J.G.	M	2.2	5.6
D.B.	M	4.4	18.9
G.W.	M	2.0	8.0
L.C.	F	3.5	16.7
F.H.	F	2.1	14.7
B.H.	F	3.3	1.3

marked, falling between 5.7 and 13.3 μg/ml for paracetamol BP and 5.1 and 16.8 μg/ml for Panasorb.

Table III shows the free paracetamol found in the urine voided in the first 24 h after ingestion. Volunteers had considerably more free paracetamol in their urine after taking Panasorb.

Table IV gives some results obtained using the method described when following the progress of some cases of paracetamol overdose.

TABLE IV

PATIENTS INGESTING A PARACETAMOL OVERDOSE

Patient	*Plasma levels (mg %)*	*Urine levels (mg %)*	*Remarks*
Female (43 years) took 50 tablets	On admission 27.8 6 h later 18.8 36 h later 1.1	1st day 195	Peritoneal dialysis containing albumen. 20 l cleared approximately 0.75 g paracetamol.
Female (23 years) took 40 tablets	12 hours after admission 5.3	1st day 20.9	
Male (28 years) access to 97 tablets	On admission 13.6 1 h later 15.4 2 h later 11.4 4 h later 8.7 8 h later 1.6		Stomach washout with 10 l of warm tap water. 5 l of 1 *N* saline and 5 % dextrose infusion over 14 h.
Female (25 years) access to 200 tablets	5 days after admission 0.8		Plasma level taken before cross circulation with husband for 10 h. None found in husband after circulation. Died 10 days after admission.

DISCUSSION

The ability of the stationary phase FFAP to separate polar compounds, such as fatty acids, indicated that paracetamol might be similarly analysed by GC. This has been substantiated and, although some tailing of the paracetamol peak occurs it is by no means unacceptable when so much time and accuracy is gained. The ratio of blood paracetamol/plasma paracetamol concentration has been established by GWILT *et al.*[3] as being 1.1. Therefore, since no major difference in paracetamol concentration occurs between plasma and red cells, plasma was taken for analysis, ether extracts of whole blood giving unsuitable gas chromatograms.

Recoveries of paracetamol from water at the 5 mg/100 ml level were quantitative when saturated with either ammonium sulphate or sodium chloride. However, when plasma was saturated with ammonium sulphate slightly better recoveries were obtained than with sodium chloride. An average recovery of 95 % was obtained from plasma, in triplicate determinations, at levels of 0.5, 1.0, 1.5, 2.0 and 20.0 mg of paracetamol per 100 ml.

Fig. 2 shows the chromatogram obtained from a blank plasma extract without the addition of the internal standard, diphenyl phthalate. Fig. 3 shows an extract from the plasma of a volunteer taking paracetamol. The unidentified peak at 13 min

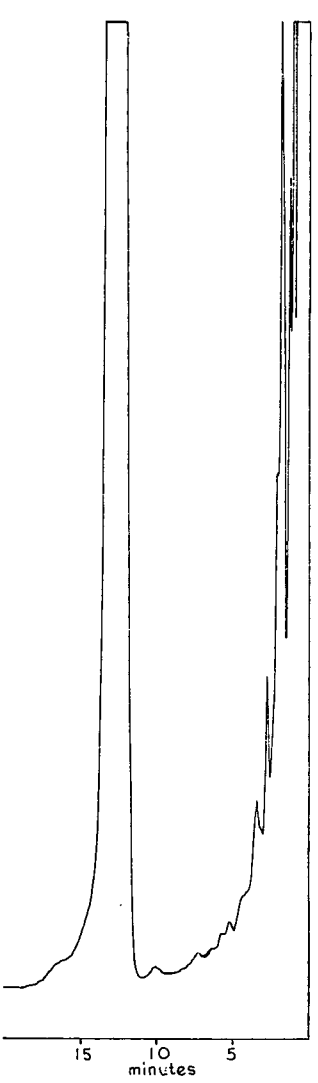

Fig. 2. The chromatogram obtained from 1 ml of a normal blank plasma without addition of the internal standard diphenyl phthalate.

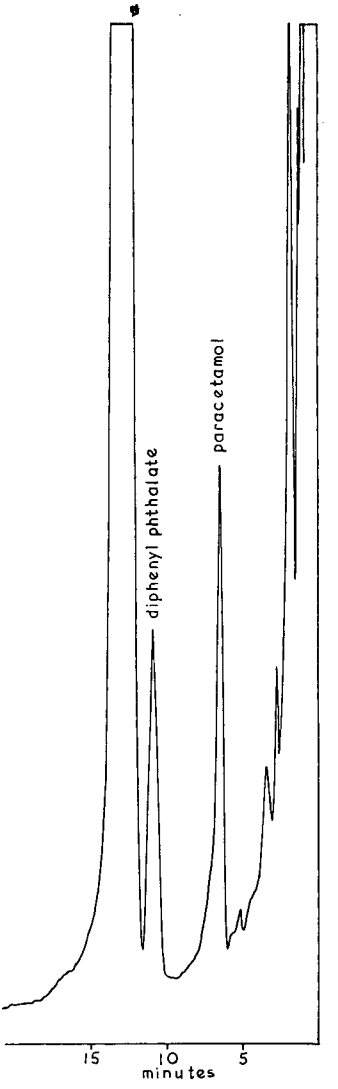

Fig. 3. The chromatogram obtained from 1 ml of plasma from a volunteer taking paracetamol.

(found in all plasma extracts), although close to the internal standard peak, can be removed by protein precipitation prior to extraction. However, the addition of another step to the analysis and the lowering of the recovery did not offer any major advantage.

The passage of urine through Florisil columns as a means of purification prior to analysis has already been noted[7,8]. When paracetamol was added to urine and passed through a Florisil column and subsequently analysed, quantitative recoveries demonstrated that the drug was not absorbed on the column. In cases of overdose, however,

when the free paracetamol level is h gh and, quite often, after the therapeutic doses when the urine is only lightly pigmented, this step may be safely omitted.

The ingestion of an overdose of paracetamol can be extremely dangerous because of the associated liver damage. The present method may be useful in correlating plasma levels with the prognosis of cases of self poisoning and may be applied to the study of regimes designed to eliminate paracetamol from the body.

ACKNOWLEDGEMENTS

The author would like to thank Dr. R. GOULDING of the Poisons Unit for his advice and interest during the course of this work, and Mr. J. SINCLAIR for his excellent technical assistance.

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REFERENCES

1 J. I. ROUTH, N. A. SHANE, E. G. ARREDONO AND W. D. PAUL, *Clin. Chem.*, 14 (1968) 882.
2 B. B. BRODIE AND J. AXELROD, *J. Pharmacol. Exp. Ther.*, 94 (1948) 22.
3 J. R. GWILT, A. ROBERTSON AND E. W. MCCHESNEY, *J. Pharm. Pharmacol.*, 15 (1963) 440.
4 R. M. WELCH AND A. H. CONNEY, *Clin. Chem.*, 11 (1965) 1064.
5 K. P. M. HEIRWEGH AND J. FEVERY, *Clin Chem.*, 13 (1967) 215.
6 J. R. GWILT, A. ROBERTSON AND E. W. MCCHESNEY, *J. Pharm. Pharmacol.*, 15 (1963) 445.
7 A. STOLMAN AND C. P. STEWART, *Analyst*, 74 (1949) 543.
8 J. GROVE AND P. A. TOSELAND, *Clin. Chim. Acta*, 29 (1970) 253.

CHROM. 5409

IDENTIFICATION DES ANTIDÉPRESSEURS TRICYCLIQUES DÉRIVÉS DE L'IMIPRAMINE PAR CHROMATOGRAPHIE EN PHASE GAZEUSE

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(Reçu le 6 avril 1971)

SUMMARY

Identification of tricyclic antidepressants derived from imipramine by gas chromatography

The identification and separation of four tricyclic antidepressants (imipramine, desipramine, clomipramine, trimeprimine) are carried out by gas chromatography. Three columns are proposed: OV–1/Igepal on Aeropak 30, XE-60/Igepal on Aeropak 30, UCON Polar on pre-alkalinized Varaport 30. The best results are obtained with the XE-60/Igepal and Ucon Polar columns. The latter has the additional advantage to allow indifferently the injection of the bases or the salts of the compounds studied in methanolic solution.

INTRODUCTION

L'importance de l'utilisation thérapeutique des imipraminiques, la fréquence et la gravité des intoxications qu'ils peuvent provoquer ont conduit de nombreux auteurs à rechercher des méthodes d'identification et de dosage de ces composés applicables aux milieux biologiques. Les techniques qualitatives, outre les réactions chromogènes d'orientation telles que celles préconisées par FORREST *et al.*[1], font intervenir essentiellement la chromatographie sur papier ou sur couche mince. Nous-mêmes avons mis au point un procédé qui assure la séparation de cinq antidépresseurs tricycliques par chromatographie sur couche mince et qui peut être appelé à rendre des services en toxicologie analytique[2]. Sur le plan quantitatif, les procédés radio-chimique sont difficilement utilisables dans la pratique courante; quant à la spectrofluorimétrie, si elle présente un intérêt certain parmi les techniques de routine (techniques colorimétriques, spectrales, ...), dû surtout à sa grande sensibilité, elle a l'inconvénient de manquer de spécificité.

Par contre la chromatographie en phase gazeuse possède l'avantage d'être facile à mettre en oeuvre et de donner des résultats à la fois qualitatifs et quantitatifs; et pourtant elle n'a été que rarement retenue pour l'identification et le dosage des imipraminiques. En 1961, GILLETTE *et al.*[3] caractérisent par ce procédé le métabolite déméthylé de l'imipramine extrait du cerveau de rat: ils se servent d'une colonne contenant 0.75 % de SE-30 (méthyl-silicone) sur Gas-Chrom P. STREET[4] propose la même phase pour mettre en évidence l'imipramine et une phase plus polaire, XE-60 (cyano-éthylméthyl-diméthyl-silicone), pour séparer les métabolites mono- et di-

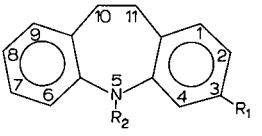
déméthylés. QUERCIA *et al.*[5,6] emploient une colonne à 2 % de SE-30 sur Aeropak 30 pour caractériser l'imipramine en solution pure ou dans ses préparations pharmaceutiques. WEDER ET BICKEL[7] séparent l'imipramine et ses principaux métabolites, préalablement extraits des tissus de rat, au moyen d'une colonne en verre contenant de l'Anakrom ABS imprégné de 1.5 % de SE-30. Enfin, plus récemment, KAZYAK ET PERMISOHN[8] indiquent trois phases stationnaires, OV-1 (diméthyl-silicone), OV-17 (phényl-méthyl-silicone) et QF-1 (trifluoropropyl-méthyl-silicone), pour la détermination des "indices de rétention" ou "indices de Kovats" (relation entre le nombre d'atomes de carbone et le logarithme du temps de rétention) de nombreuses drogues organiques, dont l'imipramine.

La spécificité et la grande sensibilité de la chromatographie gazeuse, ainsi que la rapidité de la réponse qu'elle fournit, lui permettent de satisfaire aussi bien aux exigences de l'examen toxicologique qu'à celles de l'étude pharmaco-cinétique. Aussi nous a-t-il paru opportun de l'adopter pour les déterminations que nous envisageons. Dans un premier temps notre préoccupation a été de séparer et d'identifier les cinq composés tricycliques sur lesquels s'était déjà portée notre attention. Nous exposons ici les résultats de ces recherches.

STRUCTURE CHIMIQUE DES COMPOSÉS ÉTUDIÉS

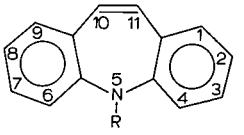
Tous possèdent un noyau tricyclique dont la partie centrale est constituée par un hétérocycle heptagonal azoté, saturé en position 10–11 (série de la dibenzodihydroazépine) ou non saturé (série de la dibenzoazépine), et une chaîne latérale de type γ-amino-propyle; dans le cas de l'opiprapol l'azote de la chaîne latérale est inclus dans un noyau pipéridinique (voir ci-après).

Serie de la dibenzodihydroazepine



	R_1	R_2
imipramine (Tofranil) chlorhydrate	H	$-CH_2-CH_2-CH_2-N(CH_3)_2$
desipramine (Pertofran) chlorhydrate	H	$-CH_2-CH_2-CH_2-NH(CH_3)$
triméprimine (Surmontil) maléate acide	H	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$
clomipramine (Anafranil) chlorhydrate	Cl	$-CH_2-CH_2-CH_2-N(CH_3)_2$

Serie de la dibenzoazepine



opipramol (Insidon) dichlorhydrate R: $-(CH_2)_3-N\langle\rangle N-CH_2-CH_2OH$

ESSAIS CHROMATOGRAPHIQUES

L'examen de la formule chimique de ces composés laisse présager, pour certains d'entre eux tout au moins (désipramine, (pipramol), des difficultés de résolution en chromatographie gazeuse. En effet, avec l(s molécules porteuses d'hydrogène mobile ou de groupement polaire, une adsorption plus ou moins réversible sur les sites actifs du support, suivie d'une désorption lente, peut provoquer la traînée des pics chromatographiques ("tailing"), ce qui diminue l'e fficacité de la colonne et fausse les résultats lorsque les analyses sont effectuées dans un but quantitatif. Différentes méthodes, visant à masquer les fonctions actives des composés injectés ou les sites réactionnels du support, peuvent être mises en oeuvre pour pallier ces inconvénients. WEDER ET BICKEL[7], par exemple, soumettent l'imipra mine N-oxyde à une réduction et les métabolites à fonction amine secondaire à une a cétylation; dans ces conditions le "tailing" n'apparaît plus, sauf dans le cas de l'hyc roxy-2-desméthylimipramine acétylée qui présente encore une adsorption non négli(able. Il est également possible de masquer les sites réactionnels des supports; le simpl(traitement de ceux-ci par un agent alcalin est souvent suffisant, comme nous l'avon s constaté encore récemment dans le cas de la nicotine[9]. Nous nous sommes donc ef orcés de trouver une ou plusieurs colonnes suffisamment sélectives pour assurer une bonne séparation des composés étudiés.

Nous avons essayé successiveme:t trois sortes de phases stationnaires, OV–1/Igepal, XE-60/Igepal, et Ucon Polai 50 HB-2000*, sur deux types de support: Aeropak 30 dans les deux premiers cas, V raport 30 dans le troisième. Les colonnes ont été préparées à l'aide de tube en aci r "inox super". Nous avons employé des taux d'imprégnation des supports par les phases relativement bas, afin de raccourcir la durée des analyses et de diminuer autan que possible la température de chauffe des colonnes. Les expérimentations ont été co duites sur un chromatographe "Aerograph 1520 B" équipé d'un détecteur à ionisat on de flame d'hydrogène, le gaz vecteur étant l'azote U. Dans tous les cas, l'une d s extrémités de la colonne était introduite dans l'injecteur de l'appareil; de la sorte es injections pouvaient être faites directement en tête de colonne. L'autre extrén ité était reliée au détecteur sans raccord capillaire. Les conditions chromatographi(ues ont été les suivantes.

(1) *OV-1/Igepal*

Nous avons opéré sur une colonne de 1.50 m de longueur, à 2 % d'OV-1 et 0.2 % d'Igepal sur Aeropak 30 (80–100 mesh). La température de la colonne était de 195°.

(2) *XE-60/Igepal*

Cette colonne, plus polaire, de 1 m de longueur, était à 4.6 % de XE-60 et 0.5 % d'Igepal sur Aeropak 30 (80–100 mesh). La colonne était chauffée à 180°.

(3) *Ucon Polar*

Nous avons également testé une colonne de 1 m de longeur, contenant 3.2 % d'Ucon 50 HB-2000 Polar sur Varaport 30 (80–100 mesh) préalablement alcalinisé

* OV-1 = diméthyl silicone; Igepal = nonyl phénoxy-polyoxyéthylène éthanol; XE-60 = cyano-éthylméthyl-diméthyl-silicone; Ucon Polar 50 Hb-2000 = polyalkylène glycols.

par 6 % de potasse. Les températures respectives de la colonne, du détecteur et de l'injecteur étaient de 205°, 215° et 220°.

Les solutions injectées dans les deux premières colonnes contenaient la base du composé étudié, solubilisée dans un solvant approprié tel que le méthanol; elles étaient préparées en agitant avec de l'éther des volumes exactement mesurés de solution aqueuse du sel convenablement alcalinisée, puis en évaporant et en reprenant le résidu par un volume déterminé de méthanol. La troisième colonne présentait l'avantage d'autoriser l'injection directe des sels en solution méthanolique.

RÉSULTATS ET DISCUSSION

Nous considérerons en premier lieu le cas de l'opipramol. Bien que possédant comme les autres substances examinées un noyau tricyclique, il s'en différencie par la présence d'une double liaison en C_{10}–C_{11} et d'une chaîne latérale terminée par une fonction alcool. L'injection de solution d'opipramol dans les trois colonnes précitées n'a provoqué aucune déflexion sur l'enregistreur, ce qui traduit une adsorption pratiquement irréversible. Ce n'est qu'en traitant la base par silylation au "BSA" [N,O-bis-(triméthylsilyl)-acétamide] préalablement à l'injection que l'élution se produit. Ces

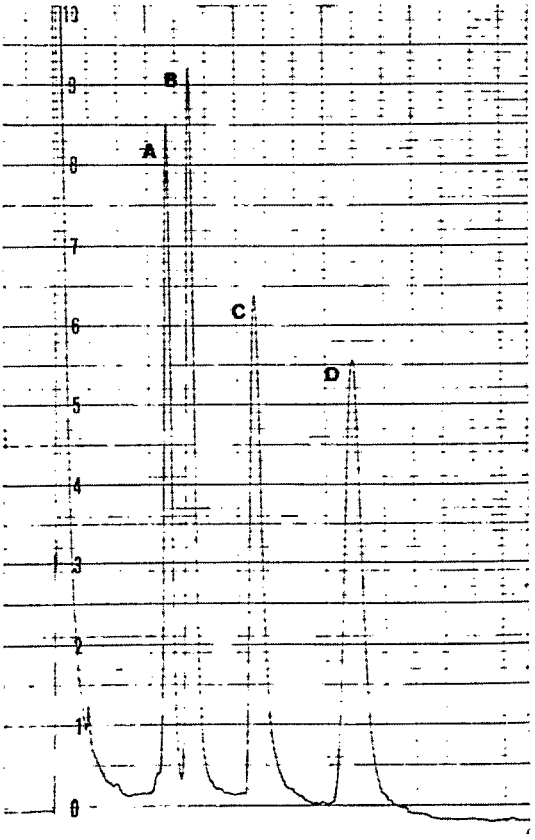


Fig. 1. Séparation sur XE-60/Igepal du mélange triméprimine (A), imipramine (B), désipramine (C) et clomipramine (D).

recherches n'ont cependant pas été poursuivies plus avant; nous avons préféré nous consacrer d'abord à l'imipramine et à ses dérivés dont l'intérêt toxicologique nous a paru supérieur à celui de l'opipramol.

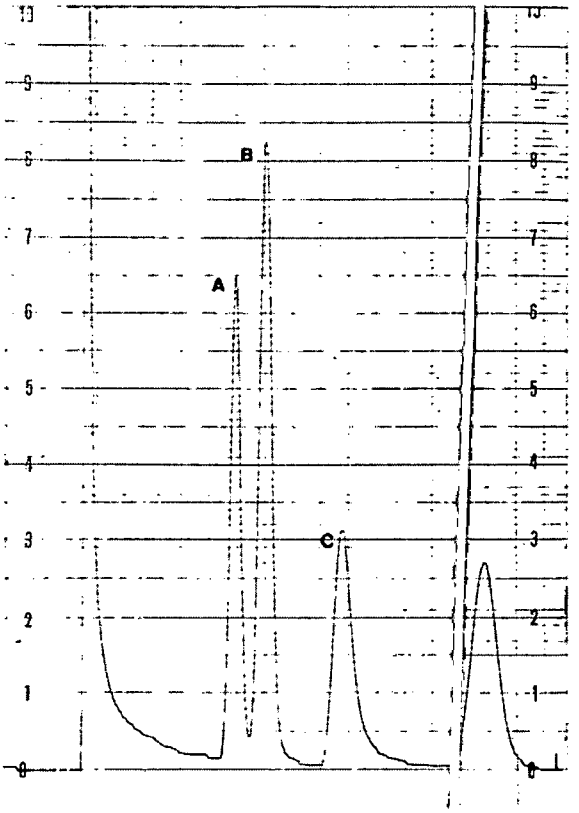


Fig. 2. Séparation sur Ucon Polar du mélange triméprimine (A), imipramine (B), désipramine (C) et clomipramine (D).

Pour les quatre autres médicaments, les performances des colonnes testées ne sont pas identiques. La séparation est très satisfaisante sur les colonnes préparées avec XE-60 et Ucon Polar, l'ordre d'élution étant toujours le même: triméprimine, imipramine, désipramine, clomipramine; les pics sont parfaitement résolus comme le montrent les Figs. 1 et 2. Avec la colonne d'OV-1, par contre, la résolution du couple imipramine–triméprimine est insuffisante (Fig. 3); l'allongement de la colonne, l'abaissement de la température et la modification du débit du gaz vecteur n'influent que modérément sur l'efficacité de la séparation. Par ailleurs les colonnes préparées avec OV-1 ou avec XE-60 ne permettent l'injection des composés que sous forme de bases, ce qui présente plusieurs inconvénients. En effet, les imipraminiques ont, à l'état de bases, une consistance huileuse; les manipulations nécessaires pour obtenir les solutions étalons ne peuvent se faire sans pertes et celles-ci, difficiles à évaluer, rendent la technique pratiquement inutilisable en analyse quantitative. C'est pourquoi nous avons préféré, pour préparer les étalons, recourir au procédé qui consiste à déplacer

la base par addition à une solution aqueuse de son sel d'un tampon de pH élevé, puis à l'extraire par un solvant organique approprié. On peut dans un premier temps, en opérant sur les bases obtenues par extraction à un pH nettement alcalin (pH 11 ou 12 par exemple), sélectionner le solvant qui semble posséder le meilleur pouvoir de solubilisation; ensuite on déterminera pour ce solvant le rendement de l'extraction en fonction du pH, après évaporation et reprise du résidu par le méthanol. Bien que la méthode paraisse donner dans l'ensemble de bons résultats, un doute subsiste quant aux possibilités de récupération totale, car le rendement ne peut pas être calculé de façon rigoureuse.

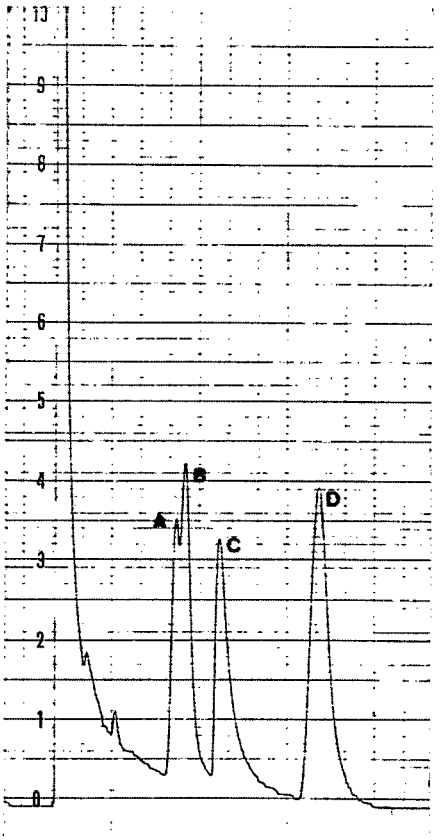


Fig. 3. Séparation sur OV-1/Igepal du mélange triméprimine (A), imipramine (B), désipramine (C) et clomipramine (D).

Un autre problème, d'ordre chromatographique, s'est également présenté avec les colonnes à base d'OV-1 et de XE-60. Alors que les amines tertiaires (imipramine, triméprimine, clomipramine) fournissent des pics parfaitement symétriques, la désipramine manifeste un phénomène de "tailing" très net. C'est pour tenter de supprimer cette traînée que nous avons essayé une colonne préparée avec une phase polaire et un support préalablement traité par un agent alcalin (Ucon Polar sur Varaport), analogue à celle déjà utilisée avec succès pour les benzophénones[10] et la nicotine[9].

Avec une telle colonne la traînée du pic de désipramine est nettement atténuée (Fig. 2) mais ne disparaît pas totalement.

Quelques enseignements pratiques peuvent être retirés de ces observations. En considérant seulement les composés sur lesquels nous avons nous-mêmes expérimenté, nous avons pu distinguer plusieurs cas:

(1) Les benzophénones, telles que la méthylamino-chloro-benzophénone ou l'amino-chloro-benzophénone provenant de l'hydrolyse acide de certains médicaments benzodiazépiniques, indifférentes au pH du milieu en ce qui concerne leur extraction par les solvants organiques, ne nécessitent pas de traitement du support chromatographique en vue d'en faire varier le pH; le seul emploi d'une phase polaire conduit à l'obtention de pics parfaitement symétriques.

(2) Pour des bases comme la nicotine ou les amphétamines, dont l'extraction par les solvants organiques ne devient complète qu'à des pH élevés (pH supérieur à 11, par exemple, pour la nicotine), l'accroissement de la polarité de la phase n'est pas suffisant pour améliorer la symétrie des pics; une alcalinisation franche du support est alors indispensable.

(3) Lorsque les substances étudiées sont extraites quantitativement à des pH moins élevés (pH 8 pour l'imipramine, pH 9 pour la désipramine, par exemple), la dissymétrie des pics ne semble apparaître qu'avec celles (telle la désipramine) dont les pH d'extraction sont les plus hauts; là encore, le "tailing" peut être considérablement réduit, non seulement en augmentant la polarité de la phase, mais aussi et surtout en alcalinisant le support. Il paraît donc se confirmer qu'il existe une relation entre le comportement chromatographique de divers types de molécules et leurs pH d'extraction par les solvants organiques à partir de milieux aqueux.

L'alcalinisation du support chromatographique, telle que nous l'avons réalisée pour la colonne à base d'Ucon Polar, rend en outre possible l'injection directe des composés à l'état de sels en solution méthanolique. Ce procédé est très avantageux car il permet un gain de temps appréciable et surtout une plus grande précision grâce aux étalons parfaitement titrés qui peuvent ainsi être préparés.

CONCLUSION

Au terme de cette étude nous disposons pour la détection des antidépresseurs imipraminiques par chromatographie gazeuse d'un ensemble de trois colonnes, différentes par la polarité de leurs phases et/ou par le pH de leurs supports. Chacune d'elles permet de réaliser l'identification spécifique de l'imipramine, de la désipramine, de la triméprimine et de la clomipramine en un temps qui, bien que ne pouvant rivaliser avec celui des réactions chromogènes d'orientation, est encore relativement court. La colonne à base d'Ucon Polar se distingue en outre par le fait qu'elle tolère l'injection des composés aussi bien à l'état de bases que sous forme de sels. Nous rapportons par ailleurs les conditions dans lesquelles elle peut être utilisée pour le diagnostic qualitatif des intoxications par imipraminiques et pour des études cinétiques et métaboliques quantitatives sur l'imipramine et la désipramine.

RÉSUMÉ

L'identification et la séparation de quatre antidépresseurs tricycliques (imipramine, désipramine, clomipramine, triméprimine) sont réalisées par chromato-

graphie en phase gazeuse. Trois colonnes sont proposées: OV-1/Igepal sur Aeropak 30, XE-60/Igepal sur Aeropak 30, Ucon Polar sur Varaport 30 préalablement alcalinisé par l'hydroxyde de potassium. Les meilleurs résultats sont obtenus avec les colonnes de XE-60/Igepal et d'Ucon Polar. Cette dernière présente en outre l'avantage de tolérer indifféremment l'injection des bases ou des sels des composés étudiés en solution méthanolique.

BIBLIOGRAPHIE

1 I. S. FORREST, F. M. FORREST ET A. S. MASON, *Amer. J. Psych.*, 116 (1960) 840 et 1021; 118 (1961) 300.
2 A. VIALA, F. GOUEZO ET C. GOLA, *J. Chromatogr.*, 45 (1969) 94.
3 J. R. GILLETTE, J. V. DINGELL, F. SULSER, R. KUNTZMAN ET B. BRODIE, *Experientia*, 17 (1961) 417.
4 H. STREET, *J. Chromatogr.*, 29 (1967) 68.
5 V. QUERCIA, C. CARDINI ET A. CALÓ, *Boll. Chim. Farm.*, 107 (1968) 383.
6 C. CARDINI, V. QUERCIA ET A. CALÓ, *J. Chromatogr.*, 37 (1968) 190.
7 H.J. WEDER ET M. H. BICKEL, *J. Chromatogr*, 37 (1968) 181.
8 L. KAZYAK ET R. PERMISOHN, *J. Forensic Sci.*, 15 (1970) 346.
9 J. P. CANO, J. CATALIN, R. BADRE, C. DUMAS, A. VIALA ET R. GUILLERME, *Ann. Pharm. Fr.*, 28 (1970) 581 et 633.
10 J. P. CANO, *Thèse Doct. Pharm. (État)*, Marseille, 1969.

CHROM. 5445

A SEARCH FOR AMINO ACIDS IN APOLLO 11 AND 12 LUNAR FINES*

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SUMMARY

Lunar fines from the Apollo 11 and 12 missions have been analyzed for amino acids and a wide range of other derivatizable organic compounds by a highly sensitive gas–liquid chromatographic method[6]. A minimum of 3 to 5 p.p.b. of each amino acid would have been detected, but there were no indications of the presence of free amino acids in the samples.

The N-trifluoroacetyl *n*-butyl ester/gas–liquid chromatographic method has been shown to give recoveries greater than 80% when samples containing 5 ng of each of seventeen amino acids were taken through the total derivatization and chromatography, thus showing that losses due to adsorptive and substrate-derivative interactions were minimal. The method was also shown capable of detecting other organic classes as higher alcohols, amines, fatty acids, and dicarboxylic acids.

The unhydrolyzed water reflux extract (17 h) of the Apollo 11 fines showed that the concentration of each of the free common amino acids were less than 4 ng/g, however, on hydrolysis of the water extract with 6 *N* HCl, a large peak was eluted on the glycine–serine region of the chromatogram, and two other peaks at 153° (80 ng/g) and 178° (40 ng/g). The large peak could be contamination as a smaller peak was observed at the same retention temperature in the parallel blank; or these peaks could be due to organic precursors which are hydrolyzed as reported by Fox[10].

The Apollo 12 unhydrolyzed water reflux extract (13 h) was also investigated, and none of the free common amino acids were detected above background (*ca.*

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4 ng/g each). However, several unidentified chromatographic peaks were observed which were not present in the corresponding procedural blank. These substances which appear to be derivatizable, are significantly above background level, and could either be indigenous to the Apollo 12 sample, the result of the unusual chromatographic effect due to unknown substances in the samples reported by us earlier[8], or contamination. An analysis of the hydrolyzed Apollo 12 water extract was not made.

Analyses were made on "clean fingerprints" which resulted in the detection of 1000 ng total of amino acids per fingerprint.

An injection port solvent-venting system has been developed which has greatly simplified the gas–liquid chromatographic analysis of nanogram amounts of amino acids[6]. This device allows the injection of the total derivatized samples (up to 100 μl) on a standard packed analytical column and prevents the solvent and derivatizing reagents from traversing the column and entering the detector. This instrumental modification will be of great value in analyses on future Apollo samples.

A series of collaborative experiments have been designed by the participating organic principal and co-investigators (PONNAMPERUMA, KVENVOLDEN, GEHRKE, FOX, HARE, HARADA; NAGY, HAMILTON; ORO) to critically evaluate and optimize the gas–liquid and classical ion-exchange methods used prior to analysis of the Apollo 14 samples. These experiments will include: (a) assessment of sensitivity of methods on a series of standard amino acids solutions at the 5, 20, and 100 ng levels by each group, and, (b) analyses for amino acids in unhydrolyzed and hydrolyzed water extracts of a single homogeneous sample of Onverwacht chert to evaluate hydrolysis, adsorption and analyses of the 20 to 60 p.p.b. levels in chert.

INTRODUCTION

The examination of extraterrestrial materials for indigenous amino acids presents possibilities for gaining information on the origin and nature of early life forms, and whether or not amino acids might be synthesized in the primitive environments of interstellar space. The lunar samples returned by Apollo flights 11 and 12 have been studied extensively in search for biologically important compounds[1–3].

The quantity of organic material present in Apollo 11 lunar samples has been reported to be quite small[1], thus requiring highly sensitive analytical methods for the detection and identification of amino acids which may be present. Further, diverse sources of possible sample contamination[4] add to the complexity of obtaining an unequivocal analysis for amino acids which might be indigenous to the lunar samples, as a single fingerprint results in 500 ng or more of amino acids.

To minimize contamination, all analyses were carried out in an ultraclean laboratory at Ames, designed specifically for lunar sample work. A highly sensitive gas–liquid chromatographic (GLC) technique developed by GEHRKE AND ZUMWALT was used[5–7] to determine whether detectible amounts of amino acids were present or absent in Apollo 11 and 12 lunar fines. With this method, the N-trifluoroacetyl (N-TFA) *n*-butyl ester derivatives are formed by esterification of the amino acids with *n*-butanol·3 *N* HCl followed by acylation with trifluoroacetic anhydride. This investigation also centered on other classes of organic compounds in addition to amino

acids that could be detected under the derivatization and chromatographic conditions used. Included were an alcohol, an amine, a fatty acid, and a dicarboxylic acid.

In this search, our major efforts were placed on the water extracts of the lunar fines, as somewhat disparate results have been reported on examination of water extracts of the Apollo 11 samples[1–3]. After aqueous reflux of the samples, the water extracts were divided, then analyzed without hydrolysis with 6 *N* HCl. Analyses were also conducted on 1 *N* HCl extracts of the lunar samples, which was the subject of an earlier report[3,8].

The aqueous extracts of Apollo 12 lunar fines have also been investigated for amino acids by classical ion-exchange techniques. NAGY *et al.*[9] reported that on analysis of three samples of lunar fines, the distribution patterns of the trace components were indicative of minor terrestrial contamination, and that *free* amino acids were not present above background levels. Studies conducted by HARADA *et al.*[10] on hydrolyzed aqueous extracts of one trench and five surface samples have shown glycine to be the principal amino acid component. Although these authors did not exclude the possibility of contamination, they reported the amino acid distribution pattern was atypical of terrestrial contamination. They did not find *free* amino acids in Apollo 12 water reflux extracts, however, on acid hydrolysis, the *total amount* for six amino acids was at the 40 to 90 p.p.b. level.

In a manuscript recently published, ZUMWALT *et al.*[6] reported the development of an instrumental-chromatographic device which has greatly simplified the analysis of nanogram quantities of amino acid derivatives by GLC. An injection port-solvent venting system permits the injection of the total derivatized sample (up to 100 μl) on a standard packed analytical column. This device prevents the large volume of solvent and acylating reagent from traversing the column and disturbing the detection system, thus greatly reducing solvent "tailing". In addition, this system eliminates the TFA interference peak observed on chromatography with ethylene glycol adipate (EGA) columns.

This paper presents the results of our investigations for amino acids in Apollo 11 and 12 lunar fines, and presents a discussion of the capabilities and limitations of analyzing for extremely small amounts of amino acids and other organics by GLC.

EXPERIMENTAL

(*I*) *Apparatus and reagents*

The gas chromatographs used in this study included two Hewlett-Packard Model 5750 instruments, each equipped with dual column oven baths, flame ionization detectors, linear temperature programmers, and Honeywell Electronik 16 strip chart recorders.

All amino acids used in this investigation were obtained from Mann Research Laboratories, Inc., New York, N.Y., or Nutritional Biochemicals Corp., Cleveland, Ohio; and were chromatographically pure. Standard amino acid N-trifluoroacetyl *n*-butyl esters were obtained from Regis Chemical Co., Chicago, Ill. The methylene chloride was obtained from Matheson, Coleman and Bell, and the *n*-butanol was a "Baker Analyzed" reagent. The trifluoroacetic anhydride was obtained from Eastman Kodak Co. The micro reaction vials are made by Analytical BioChemistry Laboratories, Inc., Columbia, Mo.

The *n*-butanol and dichloromethane were first refluxed over calcium chloride, then distilled from an all-glass system and protected from atmospheric moisture by storage in glass bottles with inverted ground-glass tops. The HCl gas for preparation of the *n*-butanol·3 *N* HCl was produced by the slow addition of concentrated hydrochloric acid to sulfuric acid, and the evolved HCl gas was passed through two sulfuric acid drying towers before bubbling into the *n*-butanol.

Triply distilled water was obtained with the use of a Kontes Model WS-2 continuous water still.

All glassware was carefully washed in detergent (Alconox) and hot water, rinsed and placed in a bath of sulfuric acid and sodium dichromate at 90–100° for at least 1 h. Each piece of glassware was copiously rinsed with singly distilled water and finally with triply distilled water and dried in an oven at 150°. The glassware was stored in covered stainless steel trays. Finally, each piece of glassware was rinsed with the solvent or reagent used in the analysis.

(II) Chromatographic columns

(A) Column I: ethylene glycol adipate on Chromosorb W, 0.65 w/w %

(1) *Materials.* Column packing I can be procured from Analytical BioChemistry Laboratories, Box 1097, Columbia, Mo. or Regis Chemical Co., 1101 N. Franklin, Chicago, Ill. EGA, stabilized grade, was obtained from Analabs, Inc., Hamden, Conn. The support material, 80/100 mesh acid washed (AW) Chromosorb W, was obtained from Supelco, Inc., Bellefonte, Pa. Acetonitrile, "Nanograde", was obtained from Mallinckrodt Chemical Works, St. Louis, Mo.

(2) *Procedure.* For preparation of 25 g of column packing I, 24.84 g of AW Chromosorb W were weighed into a 500 ml ridged round bottom flask, then anhydrous "Nanograde" acetonitrile was added until the liquid level was *ca.* 1/8 in. above the Chromosorb. 10 ml of a solution containing 16.25 mg/ml of stabilized EGA in anhydrous "Nanograde" acetonitrile were then added to the flask containing the Chromosorb W. The flask was then placed on a rotary evaporator, *slowly* removing the solvent at room temperature under a partial vacuum for *ca.* 45 min. When the Chromosorb was still slightly damp, the vacuum was increased and the flask immersed in a 60° water bath with continued rotation until the solvent was completely removed. At this point, no Chromosorb W packing should adhere to the inner wall of the flask during rotation. At the end of this period, the dry, freely-flowing column packing was poured into clean, dry 1.5 m × 4 mm I.D. glass columns with gentle tapping. Dry silanized glass wool plugs were then placed in each end of the column to hold the packing in place. Prior to analytical use, the column was placed in the gas chromatograph and conditioned at 220° with a carrier flow of *ca.* 50 ml/min of pure N_2. Analyses could be made after conditioning for 1 h when 0.5–1 μg of each amino acid were injected, however, longer conditioning times (8–24 h at 220°) were required for analyses at the nanogram level.

(B) Column II: OV-17 on H.P. Chromosorb G, 1 w/w %

(1) *Materials.* OV-17 was obtained from Supelco, Inc. Chromosorb G, H.P., 80/100 mesh was purchased from Applied Science Labs., State College, Pa. Dichloromethane, "Nanograde" was procured from Mallinckrodt Chemical Works. A solution was prepared containing 10 mg/ml of OV-17 in dichloromethane.

(2) *Procedure.* For preparation of 30 g of column packing II, 29.7 g of H.P. Chromosorb G were weighed into a 500 ml ridged round bottom flask, then "Nanograde" dichloromethane was added until the liquid level was *ca.* 1/8 in. above the support material. 30 ml of the OV-17 solution (300 mg) were pipetted into the flask. The solvent was removed in the manner described above for preparation of column packing I, and the dried column packing placed in 1.5 m × 4 mm I.D. glass columns. The columns were then placed in the gas chromatograph, and conditioned overnight at 250° with a carrier gas flow of *ca.* 50 ml/min of pure N_2. A thorough discussion of chromatographic columns for analysis of extremely small amounts of amino acids is presented in refs. 6 and 7 and includes conditioning, experimental, and storage of the column. The solvent vent-chromatographic device is available from Analytical BioChemistry Laboratories, Inc., Box 1097, Columbia, Mo.

(III) Extraction of lunar fines

(A) Sand blank

The sand blank was prepared by heating a sample of Ottawa sand (Matheson, Colman and Bell) for 48 h at 1000°.

(B) Apollo 12

A 1.2 g sample of the Apollo 12 lunar fines (ARC 12023.04) was placed in a 10 ml round bottom flask. 6 ml of triply distilled water were added, a reflux condenser was attached, and the mixture was brought to a gentle boil, then refluxed for 17 h.

At the end of this time, the flask was allowed to cool and the water extract was decanted into a 15 ml conical pyrex glass centrifuge tube. The tube was closed with a teflon lined screw cap, then centrifuged at 2700 r.p.m. for 5 min to remove fines suspended in the liquid. A slight turbidity of very fine material remained in suspension.

The water extract was again decanted into a 10 ml beaker and reduced in volume to *ca.* 1 ml by mild heating under an infrared lamp. The solution was then transferred to a micro reaction vial for derivatization to the N-TFA *n*-butyl esters.

(C) Basalt

A 1 g sample of a basalt bomb obtained from near Aloi Crater, Island of Hawaii (NASA 042070.2) was likewise refluxed with 6 ml of triply distilled water for 17 h, and the aqueous extract was taken through the entire analytical chromatographic procedure with the Apollo 12 extract to obtain a blank for the analytical method.

(D) Apollo 11

A 2.2 g sample of Apollo 11 lunar fines (10086-3 bulk A) was placed in a 50 ml pear-shaped flask, 10 ml of triply distilled water were added, and the mixture was refluxed for 17 h as described. The decanted water extract was centrifuged at 2700 r.p.m. for 5 min, then divided into two equal parts; one part was derivatized and analyzed directly by GLC, and the other part was subjected to hydrolysis with 6 *N* HCl followed by derivatization and GLC analysis.

(*E*) *Hydrolysis of water extract*

The water extract obtained from the Apollo 11 sample was evaporated to near dryness in a 10 ml beaker under an infrared lamp, then transferred with 2 ml of 6 *N* HCl into an 8 ml pyrex glass hydrolysis tube with a teflon lined cap. The sample extract was then heated in an oven for 4 h at 145° according to the procedure described by ROACH AND GEHRKE[11]. At the end of this period, the tube was removed from the oven, allowed to cool, then transferred to a micro reaction vial for derivatization and GLC analysis.

(*F*) *Reagent and performance studies*

Samples were also prepared to evaluate the purity of the reagents used in each step of the analytical procedure. The water, 6 *N* HCl, *n*-butanol·3 *N* HCl, trifluoroacetic anhydride, and dichloromethane were taken through the entire chemical and analytical procedures singly and in combinations as companion blanks for the Apollo 11 and 12 analyses. Concurrently, performance standards were prepared and analyzed to confirm the integrity of the derivatization reagents and chromatographic system.

(*G*) *Derivatization of Apollo 11 and 12 water extracts*

(1) For the conversion of amino acids to the N-TFA *n*-butyl esters, the aqueous solutions were placed in micro reaction vials, and evaporated just to dryness under an infrared lamp. Samples of larger volume were placed in 5–10 ml beakers, evaporated to *ca.* 0.5 ml, then transferred to the vial with triply distilled water. A black surface was used to support the samples, thus increasing the efficiency of the evaporative step. After all visible moisture was removed from the reaction vials, the vials were tightly closed with the teflon lined caps and allowed to cool. Then 50 μl of anhydrous dichloromethane were added, and evaporated under the infrared lamp to azeotropically remove the last traces of moisture.

(2) For esterification of the amino acids to the *n*-butyl esters, 100 μl of *n*-butanol·3 *N* HCl were added to the reaction vials. The vials were then tightly capped and placed in the ultrasonic bath just to the liquid level in the vial for *ca.* 30 sec. Water from the ultrasonic bath must be carefully excluded in this step. The exterior of the vials were dried with tissue.

(3) The vials were then placed in a 100° sand bath for 30 min. Only the lower portion of the vial containing the liquid was submerged in the sand, thus maintaining a liquid phase and allowing the sample to reflux. The samples were then removed from the sand bath and allowed to cool.

(4) After the esterification step, the samples were evaporated *just to dryness* by removing the cap from the vials, and placing them under the infrared lamp.

(5) The vials were then tightly capped, allowed to cool, then 50 μl of dichloromethane were added and evaporated under the infrared lamp as in step (1).

(6) The amino acid *n*-butyl esters were trifluoroacylated by the addition of 100 μl of TFAA–CH_2Cl_2 solution. The amount of TFAA present was varied from 0.5 to 10 μl per 100 μl of solution. The vials were again tightly capped, sonicated for *ca.* 30 sec, then heated in the 100° sand bath for 10–20 min, depending on the wall thickness of the reaction vial used. For the analysis of arginine, tryptophan, and cystine, acylation at 150° for 5–10 min yields more quantitative results. After allowing the samples to cool to room temperature, analyses were made by GLC.

(IV) Comments on the derivatization method

(A) The purity of all reagents and the cleanliness of all glassware used throughout the procedure is of obvious importance when analyzing at these low levels of amino acid concentration. The distillation of the required reagents, described in section I, must be carefully conducted. To monitor the purity of the redistilled *n*-butanol and dichloromethane, 10-ml aliquots of the redistilled products were evaporated to *ca.* 0.25 ml, then analyzed by GLC under the chromatographic conditions used for analysis of the derivatized sample. The Drierite or molecular sieve over which the distilled solvents are stored was rinsed with portions of the anhydrous reagent prior to use. All reagent bottles must be kept tightly closed and covered to exclude atmospheric moisture and air-borne dust particles.

(B) Effective techniques for obtaining clean glassware for use in the derivatization procedure include soaking the glassware in hot chromic acid cleaning solution for 4–8 h, followed by exhaustive rinsing with triply distilled water. Alternatively, the necessary glassware may be placed in a furnace and heated overnight at 500° to remove contaminating organics, and final rinsing with the reagents to be used.

(C) Moisture must be carefully excluded during the entire derivatization procedure. The *n*-butanol, HCl gas, and dichloromethane must be anhydrous, and the TFAA should not contain significant amounts of TFA.

(D) It is essential that reagent blanks be analyzed particularly often, as amino acid contamination can be easily introduced from a variety of sources, especially from fingerprints.

(E) Performance standards must be analyzed to ensure the integrity of the derivatization reagents. The concentration of the amino acids in the performance standard should be similar to the amino acid concentrations in the samples to be analyzed.

(V) GLC instrument settings and conditions

The chromatograms presented were obtained from a strip chart recorder with 1 mV full scale response. The amplification of the signal from the hydrogen flame detector was accomplished by use of an electrometer with a maximum sensitivity of 1×10^{-12} A/mV. The designation $\times 1$ appearing on the figures denotes this sensitivity as maximum. Other sensitivity designations (*e.g.* $\times 2$, $\times 2.5$, etc.) represent changes in sensitivity by that particular factor. The changes in sensitivity during the analyses were made to obtain the optimum amount of information, although they do not add to the convenience of interpretation. The symbols ADJ appearing on the chromatograms signify changes in the suppression amperage only and do not alter the sensitivity setting of the analysis.

RESULTS AND DISCUSSION

Initial studies included the analysis of blanks of the materials and reagents used for the GLC analysis of amino acids. A chromatographic column blank is presented in Fig. 1, with no sample injected. The extraneous peaks that were eluted above 200° did not significantly interfere with the analysis for amino acids, as only the more complex protein amino acids were eluted above this temperature, *i.e.* tyrosine, glutamic acid, lysine, arginine, tryptophan, and cystine. Interest was primarily

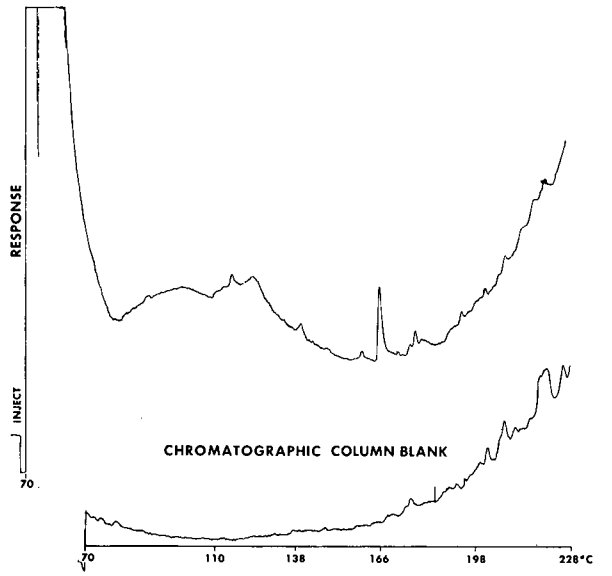

Fig. 1. Chromatographic column and reagent blank. 100 μl *n*-butanol·3 *N* HCl and 100 μl TFAA–CH_2Cl_2 (0.5 μl/200 μl). Final volume, 50 μl; injected, 5 μl; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 228°; attenuation, 4 × 10^{-12} AFS; injection port, 280°. Column, 1.0 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

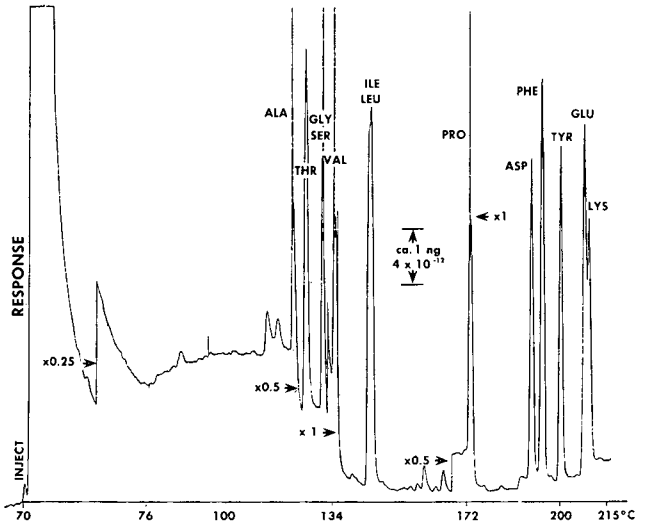

Fig. 2. Performance-chromatography standard. Derivatization and GLC analysis of standard amino acid mixture. Derivatized, 1 μg of each; injected, 25 ng of each; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 215°; attenuation, 16 × 10^{-12} AFS; injection port, 280°. Column, 1.0 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

centered on alanine, glycine, threonine, serine, and aspartic acid, as small amounts of these and other less complex compounds were reported in samples of Apollo 11 lunar fines by other investigators[1,2]. Also, Fig. 1 presents the reagent and chromatographic blank used for derivatization and analysis of amino acids as their N-trifluoroacetyl *n*-butyl esters. The small peak eluted at 166° did not correspond in retention temperature to any of the amino acid derivatives.

A derivatization performance study was then conducted to confirm that the employed derivatization reagents were in sufficient molar excess to quantitatively derivatize the amino acids. This information was of particular importance as the amount of TFAA used to acylate the samples was deliberately kept small, in order to suppress the TFA interference peak when chromatographing on the ethylene glycol adipate column for confirmation of any amino acids which might be found. Therefore, a relatively large amount (1 μg) of each amino acid was used to evaluate the acylating

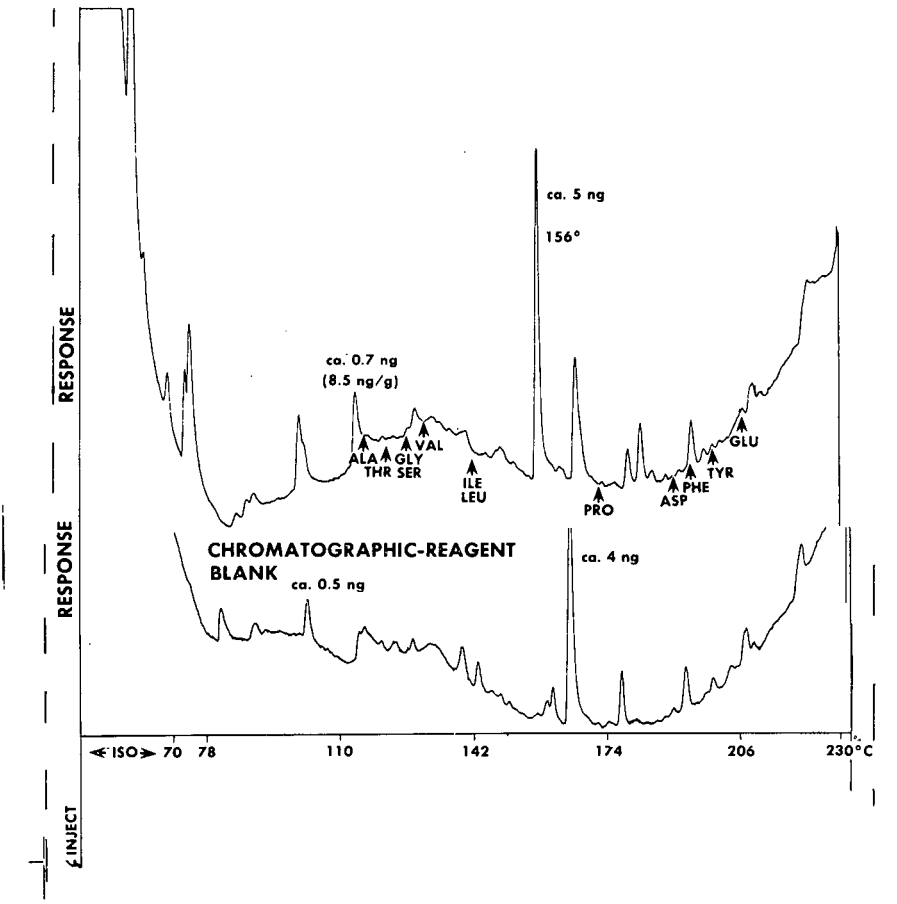

Fig. 3. Apollo 11 water extract; 3 × distilled water, reflux 17 h. Sample, 1.1 g of lunar fines (3.3 ml). 100 μl *n*-butanol·3 *N* HCl and 100 μl TFAA–CH_2Cl_2 (0.5 μl/100 μl). Final volume, 60 μl; injected, 5 μl; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 232°; attenuation, 4 × 10^{-12} AFS; injection port, 280°. Column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

reaction of the TFAA–dichloromethane solution. The derivatization reagents were found to be suitable, yielding large peaks for 25 ng of each amino acid, as seen in Fig. 2. Several attenuation changes were made during this analysis to obtain information on the instrumental sensitivity. 1 ng gave *ca.* a 10 % full scale response at an attenuation setting of 4×10^{-12} AFS. The derivatization was found to be essentially quantitative, as seen by dilution and analysis of standard amino acid mixtures.

The water extract of the Apollo 11 sample was then examined. The extract was divided equally, one part derivatized and analyzed directly, and the remaining part hydrolyzed with 6 *N* HCl at 145° for 5 h then taken through the derivatization and GLC procedures. Concurrently, complete reagent blanks were prepared as parallel samples, and were analyzed under the same experimental and chromatographic conditions as the derivatized lunar extracts.

Fig. 3 presents the chromatograms obtained on analysis of the water extract

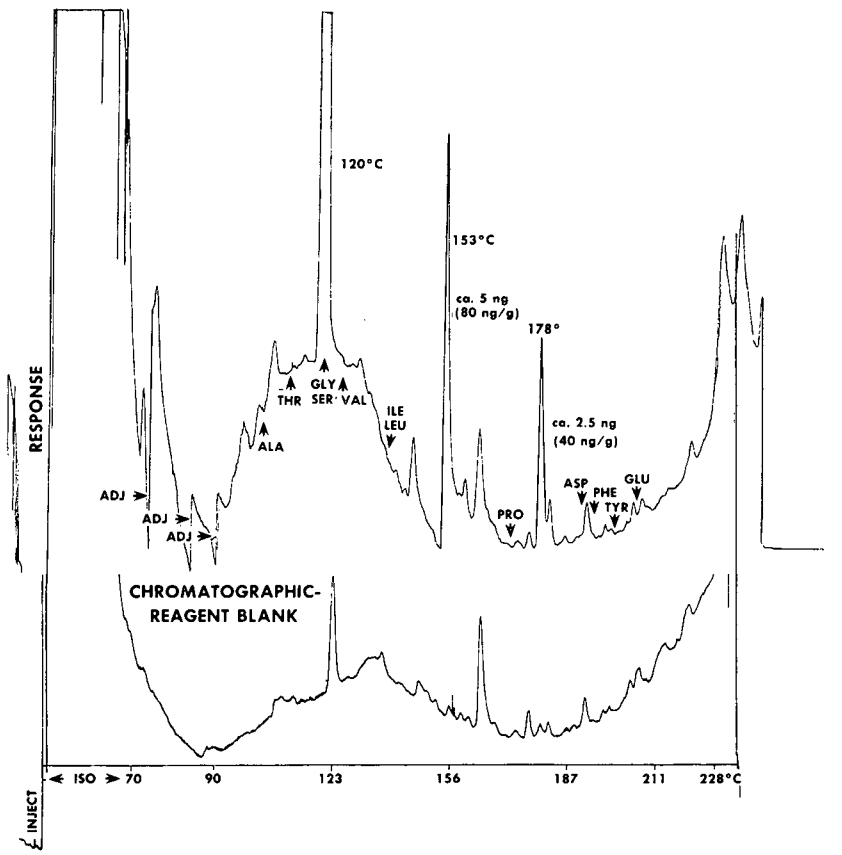

Fig. 4. Apollo 11 water extract, hydrolyzed; 3 × distilled water, reflux 17 h. Sample, 1.1 g of lunar fines (3.3 ml), hydrolyzed with 6 *N* HCl, 5 h at 145°. 100 μl *n*-butanol·3 *N* HCl and 100 μl TFAA–CH_2Cl_2 (0.5 μl/100 μl). Final volume, 80 μl; injected, 5 μl; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 232°; attenuation, 4×10^{-12} AFS; injection port, 280°. Column, 1.0 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

corresponding to 1.1 g of the Apollo 11 sample, and the companion chromatographic-reagent blank. These analyses show that less than 4 ng of each amino acid were present per g of lunar material.

The analysis of the hydrolyzed water extract, also corresponding to 1.1 g of Apollo 11 fines, resulted in the chromatogram presented in Fig. 4, along with the companion chromatographic-reagent blank. Again, no amino acids were found above the 4 ng/g level. The large peak eluted at 120° occurred in the glycine–serine region of the chromatogram, therefore no conclusions could be drawn regarding these amino acids. A smaller peak eluted at the same temperature was observed in the corresponding reagent blank. The peaks that were observed at 153° and 178° exhibited retention times distinctly different from the amino acid derivatives, and could not be characterized.

The chromatogram obtained on analysis of the derivatized water extract of 1.2 g of Apollo 12 lunar samples is shown in Fig. 5. Concurrently, an equivalent amount of basalt was extracted with water under the same conditions (13 h under reflux), then derivatized and analyzed to serve as a complete reagent and procedural blank of the method. The Apollo 12 water extract gave no indication of containing indige-

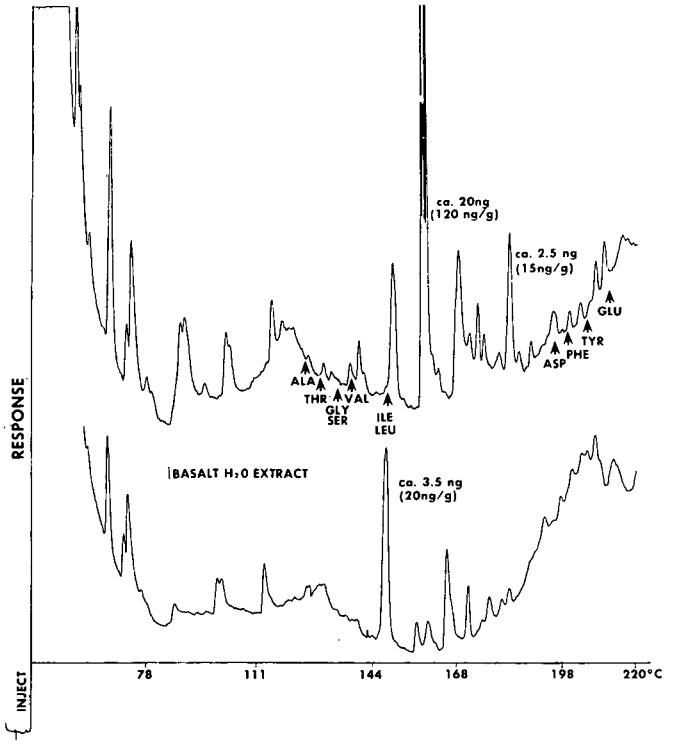

Fig. 5. Apollo 12 water extract; 3 × distilled water, reflux 13 h. Sample, 1.2 g of lunar fines (6 ml). 100 μl of *n*-butanol·3 *N* HCl and 100 μl TFAA–CH_2Cl_2 (0.5 μl/100 μl). Final volume, 30 μl; injected, 5 μl; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 220°; attenuation, 4 × 10^{-12} AFS; injection port, 280°. Column, 1.0 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

nous amino acids at a level greater than 5 ng/g *each* in the lunar soil. The large peak eluted at 156° (corresponding to *ca.* 120 ng/g) was not identified.

Analyses of the water extracts of the returned lunar samples (Apollo 11 and 12) resulted in none of the common amino acids being detected at concentration levels (as *free* amino acids) of *ca.* 4–5 ng/g. However, several unidentified chromatographic peaks were observed, particularly in the Apollo 12 sample, which were not present in the corresponding procedural blanks. These substances which appear to be derivatizable, could either be indigenous to the sample, contamination, or the results of the unusual chromatographic effect reported earlier by these authors[8].

At the 2nd Lunar Science Conference, Houston, January, 1971, Fox and coworkers[10] reported that they did not find *free* amino acids in Apollo 12 fines at the nanogram level, however, they did find amino acids after 6 *N* HCl hydrolysis of the aqueous extract. Nagy *et al.*[9] reported at this conference that *free* amino acids in Apollo 12 were not present at a level greater than background. A major peak at 120° was observed in the Apollo 11 *hydrolyzed* water extract. Two other major peaks were also observed at 153 and 178°. These may be due to the observation reported by Fox. Further comfirmation is needed.

To evaluate other classes of organic compounds in addition to amino acids that could be detected by the derivatization and chromatographic methods used, a synthetic solution containing an alcohol, an amine, a fatty acid, a dicarboxylic acid, and an amino acid was studied. Fig. 6 presents the chromatogram obtained after derivatization and chromatography of the standard, indicating that these classes of compounds were amenable to detection by the analytical method used.

Of interest in this search was a knowledge of the amount of amino acids which

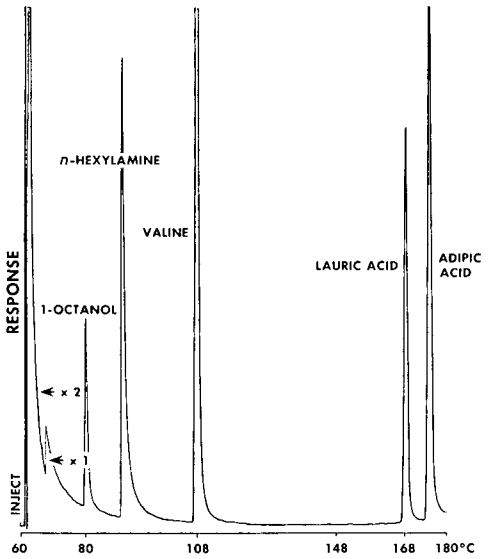

Fig. 6. Performance and chromatographic standard. Derivatization and GLC analysis of a five-component mixture. Sample injected, *ca.* 1 μg of each; attenuation, 2×10^{-10} AFS = × 1; initial temperature, 60°, program rate, 6°/min; final temperature, 180°; injection port, 280°. Column, 3.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb W, 1.5 m × 4 mm I.D. glass.

might be expected to originate on contamination by a *clean single* fingerprint. The GLC analysis of fingerprint amino acids was presented in an earlier manuscript[6], and showed that a minimum of *ca.* 1 μg of *total* amino acids could be expected per "clean" fingerprint (Fig. 7). HAMILTON[12] had earlier investigated contamination from amino acids resulting from thumbprints by classical ion-exchange chromatography and reported that the detection of each of seventeen amino acids ranged from *ca.* 500 to 20 ng per dry thumbprint. He reported that serine and glycine were the most abundant amino acids resulting from wet thumbprints with *ca.* 10000 and 7000 ng, respectively.

Injection port solvent vent-chromatographic system

Analyses of samples containing submicrogram quantities of seventeen protein amino acids were successfully conducted, using the derivatization procedure described in the EXPERIMENTAL section and the injection port solvent vent-chromatographic system. Fig. 8 shows the chromatogram obtained after an aqueous solution containing 5 ng of *each* amino acid was taken through the *total* derivatization and GLC chromatographic method. The derivatization and instrumental conditions are given in the figure legend.

CONCLUSIONS

The examination of unhydrolyzed water reflux extracts of Apollo 11 and 12 lunar fines by a highly sensitive gas–liquid chromatographic method resulted in none of the common amino acids being identified at a concentration of *ca.* 3 to 5 ng/g of each. These observations are in agreement with the results reported by NAGY AND

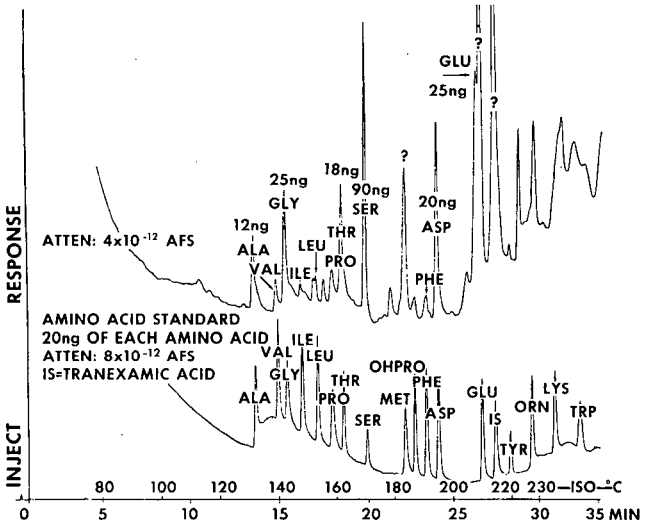

Fig. 7. GLC analysis of a fingerprint. Amino acid N-TFA *n*-butyl esters. Final sample volume, 60 μl; injected, 45 μl; vent time, 45 sec; initial temperature, 55°; program rate, 6°/min; final temperature, 230°. Column, 0.65 w/w % EGA on 80/100 mesh Chromosorb W, with solvent venting system.

HAMILTON[9] and HARADA *et al.*[10], using classical ion-exchange methods in that *free* amino acids were not found above background levels.

At this date, the only divergence of results obtained by the various investigators is the presence or absence of amino acid precursors in water reflux extracts of lunar fines, which yield some amino acids on hydrolysis with 6 *N* HCl. FOX and co-workers[10] have reported the presence of amino acids precursors in the Apollo 12 fines which gave rise on hydrolysis to a *total* of 20 to 90 ng/g of amino acids. NAGY AND HAMILTON did not report results on hydrolysates of the aqueous extracts. Although we did not confirm the findings of FOX, several prominent non-amino acid peaks were found in the chromatograms obtained *after hydrolysis* of the water extract of an Apollo 11 sample. In particular, a large unidentified peak occurred in the glycine–serine region (*ca.* 120 ng/g) and two other peaks were observed at 153° (80 ng/g) and 178° (40 ng/g). An acidic hydrolysate of the aqueous extract of Apollo 12 fines was not made. Further confirmation experiments will be necessary byGLC, GLC–MS, and classical ion-exchange (CIE) for an unequivocal conclusion as to the formation of amino acids on hydrolysis of precursors.

Based on the reports of Apollo 11 and 12 investigations, the unequivocal de-

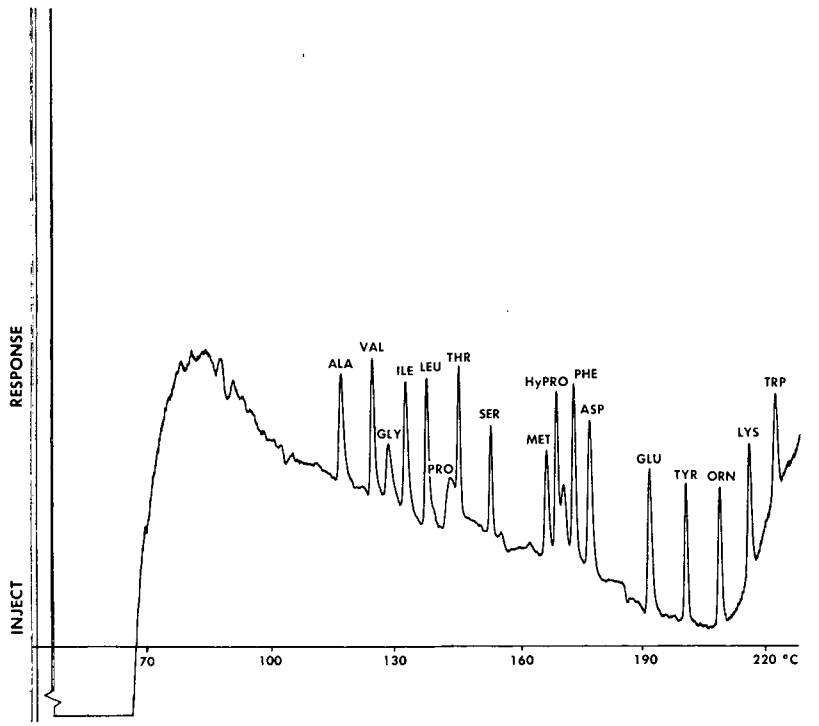

Fig. 8. Derivatization and GLC analysis of 5 ng of each amino acid. Derivatization: esterification, 25 μl *n*-butanol·3 *N* HCl at 100° for 70 min; acylation, 25 μl CH_2Cl_2–TFAA (9:1) at 100° for 20 min. GLC analysis: mode, dual differential; injection port solvent vent. Sample injected, 25 μl; solvent vent time, 30 sec; injection port temperature, 150°; initial temperature, 70°; initial hold, 4 min; program rate, 6°/min; attenuation, 8 × 10^{-12} AFS. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Precolumn, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm.

termination of the existence of indigenous amino acid precursors in lunar samples is a formidable task, as the concentration of these compounds, if present, is extremely small. Therefore, with this objective in mind, a series of interlaboratory experiments has been designed by the participating investigators, PONNAMPERUMA, KVENVOLDEN, GEHRKE; FOX, HARE, HARADA; NAGY, HAMILTON; ORO; and FLORY) to thoroughly evaluate and optimize the GLC–MS and CIE analytical methods used to detect and elucidate the structure of these compounds. This study will be conducted in advance of, and will be aimed at, obtaining unequivocal results from Apollo 14 analysis. These experiments will include: (a) an assessment of sensitivity of methods on a series of standard amino acid solutions at the 5, 20, and 100 ng levels by each group, using both CIE and GLC methods, and, (b) analyses for amino acids in hydrolyzed and unhydrolyzed water extracts of a single homogeneous sample of Onverwacht cherts to determine presence of precursors. These studies will aid in determining the most useful techniques for extraction, hydrolysis, and detection of the amino acids in samples of Apollo 14.

REFERENCES

1 B. NAGY, C. N. DREW, P. B. HAMILTON, V. E. MODZELESKI, MARY W. SISTER MURPHY, W. N. SCOTT, H. C. UREY AND M. YOUNG, *Science*, 30 (1970) 770.
2 S. W. FOX, K. HARADA, P. E. HARE, G. HINSCH AND G. MUELLER, *Science*, 30 (1970) 767.
3 C. W. GEHRKE, R. W. ZUMWALT, W. A. AUE, D. L. STALLING, A. DUFFIELD, K. A. KVENVOLDEN AND C. PONNAMPERUMA, *Geochim. Cosmochim. Acta, Suppl., 1*, No. 2, (1970) 1845.
4 D. A. FLORY, B. R. SIMONEIT AND D. H. SMITH, *Apollo 11 Organic Contamination History*, NASA Tech. Rep., 1969; D. A. FLORY AND B. R. SIMONEIT, *A Summary of the Apollo 11 Organic Contamination History*, NASA Tech. Rep., 1969.
5 R. W. ZUMWALT, D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 53 (1971) 171.
6 R. W. ZUMWALT, K. KUO AND C. W. GEHRKE, *J. Chromatogr.*, 57 (1971) 209.
7 C. W. GEHRKE, K. KUO AND R. W. ZUMWALT, *J. Chromatogr.*, 57 (1971) 193.
8 C. W. GEHRKE, R. W. ZUMWALT, W. A. AUE, D. L. STALLING AND J. J. RASH, *J. Chromatogr.*, 54 (1971) 169.
9 B. NAGY, C. N. DREW, P. B. HAMILTON, J. E. MODZELESKI, V. E. MODZELESKI, L. A. NAGY AND H. C. UREY, *2nd Ann. Lunar Sci. Conf., Houston, Texas, January, 1971.*
10 K. HARADA, P. E. HARE AND S. W. FOX, *2nd Ann. Lunar Sci. Conf., Houston, Texas, January 1971.*
11 D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 52 (1970) 393.
12 P. B. HAMILTON, *Nature*, 205 (1965) 284.

CHROM. 5417

MULTI-SAMPLE QUANTIFICATION OF AMINO ACIDS AND IMINO ACIDS WITH A SINGLE ANALYTICAL SYSTEM

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SUMMARY

An automated procedure is described for quantifying amino acids, imino acids, and other ninhydrin-positive compounds which is based on making absorbancy measurements at the single wavelength of 405 mμ. Experimental data indicate that the sensitivity of the modified capability is equal to or greater than that obtained using a conventional procedure. Additional evidence is presented that validates a further methodologic development which makes possible the analysis of three samples with the instrumentation designed for the analysis of only one sample.

INTRODUCTION

A voluminous literature concerning automated amino acid methods has accumulated since the first automatic recording system described by SPACKMAN *et al.*[1]. Notwithstanding the many noteworthy instrumental and procedural modifications, there is at least one aspect of the original method which has not been altered; namely the measurement of the ninhydrin chromogens of amino acids and imino acids at wavelengths of 570 mμ and 440 mμ, respectively. Accordingly, most commercial analyzers are equipped with at least two colorimeters and a dual recording system.

The present report describes what appears to be the first departure from the conventional use of two and three colorimetric recording systems. As described, the present procedure not only facilitates the use of a single recording system with optimum sensitivity for quantifying imino acids and amino acids, but as a consequence thereof, makes possible the simultaneous analysis of three samples with the instrumentation designed by its manufacturer to analyze only one sample at a time.

MATERIALS AND METHODS

A Technicon amino acid autoanalyzer of the type available in 1967 was used. Of particular note here is the fact that the analyzer was originally equipped with a two-coil heating bath, three colorimeters, and a three-point recorder. With respect to the colorimeters, one housed a 15 mm flowcell and a set of 440 mμ interference filters, one had a 15 mm flowcell and a set of 570 mμ filters, and the other one hand an

8 mm flowcell and a set of 750 mμ filters. Additionally, the manifold assembly consisted of the four tubes recommended by the manufacturer[2].

In accordance with the procedure described below, the following instrumentational modifications were made: (1) a third glass coil was placed within the heating bath used for color development; (2) two additional glass columns (140 × 0.636 cm) were packed to a height of 130 cm with Technicon Chromobeads, Type B; (3) the water jackets surrounding the chromatographic columns were connected in tandem so that all three columns were thermostatically controlled by a single water bath; (4) two additional sets of manifold tubing were placed along with the original set in the two-level end-blocks used with the proportioning pump; (5) 15 mm flowcells and sets of 405 mμ filters were placed within each of the three colorimeters; and (6) a four-arm metal connection (Swagelock "cross", Crawford Fitting Company, Solon, Ohio) was so placed on the outlet side of the high-pressure eluent pump that eluent buffer entered through one arm of the cross and was dispensed through the other three arms. Appropriate lengths of high-pressure Teflon tubing (1/16 × 0.03 in.) were used to connect the three outlets to the flanged Teflon fitting at the top of the three columns. In essence, the latter modification made possible the simultaneous elution of three columns using a single eluent pump and a single Technicon Autograd. The eluent pump rate was adjusted to deliver a total of 1.50 ml/min.

Two other departures from the suggested procedure[2] dealt with the production of the buffer gradient. One modification was the use of a previously reported gradient[3]. In brief, the gradient was produced by placing 60 ml of a citrate buffer (pH 2.88) in each of the first five chambers of the Autograd, and 60 ml of another citrate buffer (pH 5.00) in each of the four remaining chambers. The total volume of the resulting buffer gradient was sufficient for operating a single column system. With the novel three-column analyzer described herein, however, each of the nine chambers of the Autograd was filled to capacity; *i.e.*, each contained 160 ml of buffer.

Two operational procedures were used with the modified analyzer. In one procedure, all components were used for the separation and quantification of a known

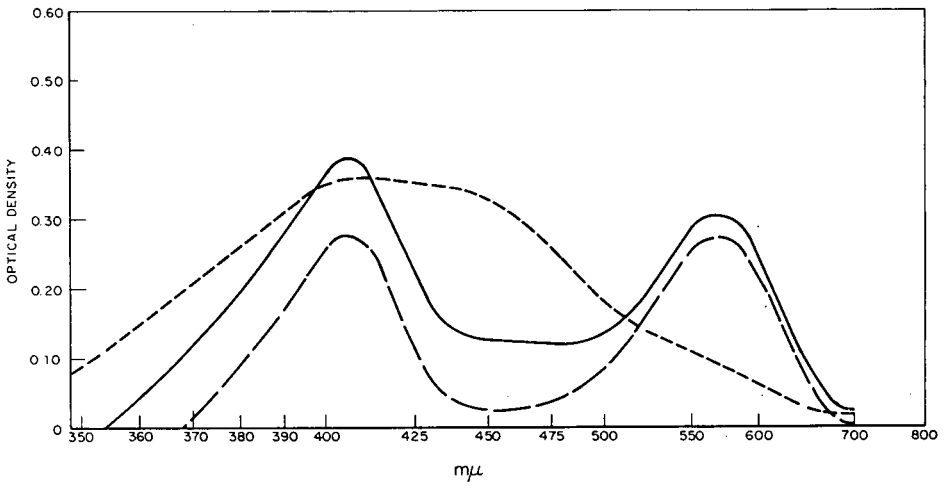

Fig. 1. Absorption spectra of the ninhydrin products of cystine (———), threonine (– – – –) and proline (------).

mixture of amino acids and imino acids. The other procedure was the aspiration of individually prepared solutions of amino acids and imino acids through the manifold tubing normally used for aspirating column effluent. With respect to the latter procedure, a set of calibration standards was prepared for each compound in 0.1 *N* HCl. Compounds examined in this study included the following: α-alanine, ammonium sulfate, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine. Each set of standards was aspirated for a sufficient period of time to permit the recording of a plateau at maximum absorbance. Additionally, the repumped reactant fluid from each standard was not discarded; instead, each was collected and subjected to spectral analysis utilizing a Beckman Model DK-2 ratio-recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

The ninhydrin reagent and other solutions used with the analyzer were prepared in accordance with instructions furnished with the analyzer[2].

RESULTS

The rationale for quantifying ninhydrin chromogens of imino acids, amino acids, and closely related compounds with a single recording system is clearly demonstrated by the spectral data shown in Fig. 1. The spectral curves for the proline, cystine and threonine chromogens are shown to represent the three general types of spectra found among the 21 compounds examined. Spectra of the proline type were

TABLE I

RELATIVE ABSORBANCE OF NINHYDRIN CHROMOGENS

Compound	*Relative absorbance*[a]
Ammonium sulfate	1.00
Leucine	1.01
Isoleucine	1.02
Arginine	1.03
Threonine	1.03
Glycine	1.04
Alanine	1.04
Methionine	1.04
Tyrosine	1.04
Phenylalanine	1.04
Serine	1.05
Tryptophane	1.05
Aspartic acid	1.05
Valine	1.06
Histidine	1.11
Ornithine	1.15
Glutamic acid	1.21
Lysine	1.24
Cystine	1.36
Proline	1.06
Hydroxyproline	1.09

[a] Values are $A_{405}:A_{570}$ for all compounds except proline and hydroxyproline which are $A_{405}:A_{440}$.

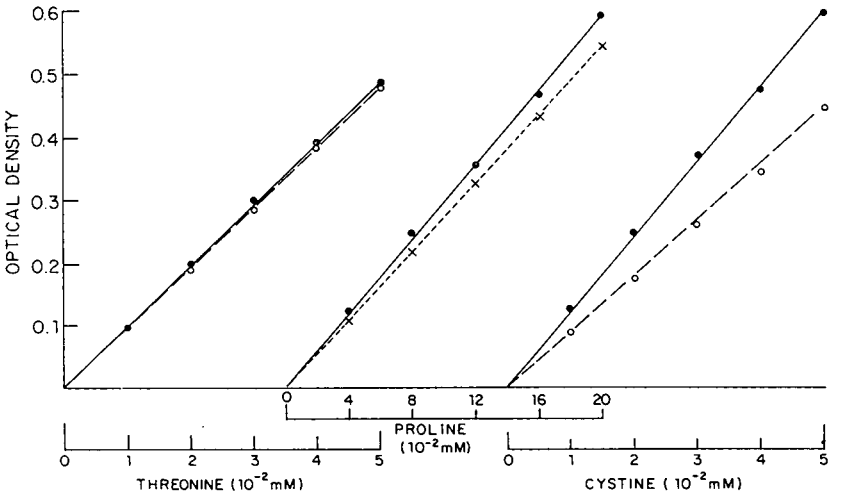

Fig. 2. Calibration curves for the ninhydrin products of threonine, proline and cystine (from left to right). Absorbance measurements for threonine and cystine products were made at 405 mμ (●—●) and 570 mμ (○---○); absorbancies for proline chromogen measured at 405 mμ (●—●) and 440 mμ (×---×).

clearly different from those of the cystine and threonine types, being characterized by the single, broad-absorption band. Differentiation between the other two spectral groups was less clear-cut as they were based on the relationship of absorbance at the two maxima, *i.e.*, at 405 mμ and 570 mμ. With respect to spectra of the threonine type, the absorbance values at the two wavelengths were virtually identical. This was not the case for spectra of the cystine type as the absorbance at 405 mμ was substantially higher than that at 570 mμ.

The data presented in Table I offer a summary of the spectra of the 21 ninhydrin chromogens. A noteworthy finding was that absorbancies at 440 mμ for imino acids and at 570 mμ for amino acids were less than absorbancies at 405 mμ.

The validity of making absorbance measurements at 405 mμ is demonstrated by the calibration data presented in Fig. 2. For brevity, the categorization employed above for absorption spectra is used for illustrating the linearity of absorbance measurements. These data not only substantiate the finding of equal or greater absorbance at 405 mμ, but, of even greater importance, indicate the quantitative soundness of using a single wavelength for quantifying the diverse ninhydrin chromogens.

As evidenced by the data shown in Table II, the separation and quantification of a mixture of amino acids further validated the use of the single recording system. Actually, these data were obtained from an operational procedure which differed from that suggested by the manufacturer[2] in one noteworthy respect; namely, a 15 mm flowcell and a set of 405 mμ filters were placed in the colorimeter which had originally been equipped with an 8 m flowcell and a set of 570 mμ filters. It is apparent from the data in Table II that not only was there less variation in area measurements at 405 mμ, but there was also a consistent gain in sensitivity. As might be expected from the data presented in Table I, the gain in sensitivity was especially marked for cystine, glutamic acid, lysine and histidine.

TABLE II

ANALYSIS OF CHROMATOGRAPHIC PEAKS BY DUAL AND SINGLE RECORDING SYSTEMS

Compound	*Area constants* ($H \times W/\mu mole$)			
	Conventional recording system[a]		*Modified recording system*[b]	
	Mean ($n = 3$)	*Coefficient of variation*	*Mean* ($n = 3$)	*Coefficient of variation*
Aspartic acid	51.77	1.40	54.39	1.75
Threonine	52.93	1.94	55.10	1.96
Serine	53.87	1.67	55.85	1.10
Glutamic acid	51.80	2.51	63.76	2.17
Proline	13.63	7.35	14.98	4.38
Glycine	58.00	2.15	60.49	1.29
Alanine	51.07	2.16	53.34	2.64
Valine	48.63	2.22	51.71	1.51
1/2-Cystine	29.90	1.67	43.06	1.60
Methionine	53.90	2.60	57.12	2.28
Isoleucine	50.67	1.68	52.84	0.95
Leucine	57.87	1.93	59.04	0.87
Tyrosine	55.93	1.74	58.78	1.14
Phenylalanine	56.00	1.64	58.91	1.89
$(NH_4)_2SO_4$	26.17	3.98	28.77	2.68
Lysine	57.77	2.14	69.72	1.92
Histidine	59.07	2.13	65.21	1.04
Arginine	55.93	0.88	59.00	0.78
Mean		2.32		1.77

[a] Values based on absorbance at 570 mμ except for proline which was measured at 440 mμ.
[b] All values based on absorbance at 405 mμ.

The data presented in Table II were based on the analysis of 50 μl of a standard mixture in which each amino acid was present in a concentration of 2.5 mM except for the 1.25 mM cystine. Data obtained from replicate analyses of 10, 20, 30, and 40 μl of the same mixture revealed linear responses that were similar to those shown in Fig. 2.

After validating the use of the single colorimetric recording system for amino acids, imino acids and other ninhydrin-positive substances, efforts were made to utilize the triple colorimetric recording capability of the analyzer for the simultaneous analysis of three samples. In the latter endeavor, special efforts were made to utilize only the instrumental components which therefore had been used for the analysis of a single sample. It was recognized that certain expendable components such as manifold tubing, additional chromatographic columns and resin would be essential. It was desirable, however, that no additional instrumentation such as eluent and proportioning pumps, heating and circulating water baths, Autograds, colorimeters and recorders would be necessary.

The schematic drawing shown in Fig. 3 illustrates the basic features of the resulting three-sample analyzer. Although the use of the single colorimetric system described above was the most important factor leading to the desired operational procedure, a previously reported methodologic development proved to be a decisive factor. The latter factor was the modified buffer gradient[3] which, among other favorable features, made possible the elution of chromatograms with a substantially

reduced total volume of eluent buffer. The importance of the gradient volume is discussed below along with other aspects of the present procedure.

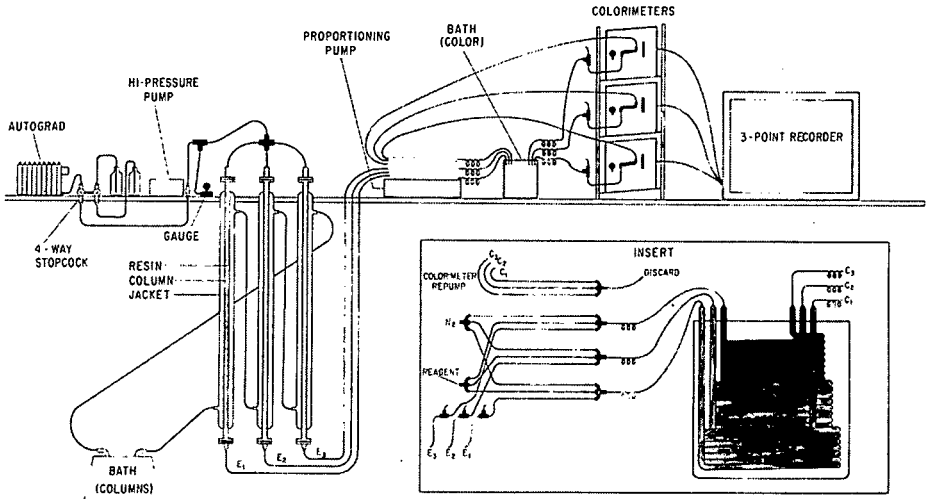

Fig. 3. Schematic drawing of 3-sample amino acid analyzer. Insert shows close-up view of manifold and glass coils contained within the heating bath used for color development. E_1, E_2 and E_3 denote effluents from the three columns. C_1, C_2 and C_3 denote the reactant fluids derived from effluents E_1, E_2 and E_3, respectively. See text for function and use of the individual components of the analyzer.

DISCUSSION

The present finding of two absorption maxima in the visible spectrum for ninhydrin products of amino acids is not unprecedented. Absorption maxima at 408 mμ and 578 mμ for the ninhydrin chromogen of alanine were reported by MEYER[4]. While those maxima are slightly higher than the 405 mμ and 570 mμ reported here, the slight shifts might be readily explained on the basis of procedural differences, particularly in the preparation of the ninhydrin reagent. As far as can be ascertained, however, advantage has not heretofore been taken of the 408 mμ (405 mμ) absorption maximum. The report of the first automatic amino acid analyzer[1] appeared a year after the report of MEYER[4]. The equipping of the three colorimeters in that original automated method necessitated two absorbancy measurements at 570 mμ and one measurement at 440 mμ. Moreover, it appears that all commercial automatic amino acid analyzers marketed since that time have retained that feature of the methodology.

In addition to the obvious advantage of using a single colorimetric recording system, the present data suggest that a more sensitive assay of imino acids as well as amino acids is obtained by absorbancy measurements at 405 mμ. The finding that the maximum absorbance of the ninhydrin products of proline and hydroxyproline was closer to 405 mμ than to 440 mμ was somewhat surprising as the latter wavelength has seemingly been universally accepted as the absorbance maximum.

The discovery that one colorimeter could be used to quantify ninhydrin-positive compounds was the most decisive factor in establishing the three-sample analyzer;

however, two other factors were important. One factor was to ensure that the eluent pump rate of 1.5 ml/min was uniformly distributed so that each column would be eluted at the rate of 0.50 ml/min. Two steps were necessary to achieve uniform elution rates. The first step was to pack the three columns in an identical manner. This was accomplished by the simultaneous filling of the columns with a single, homogeneous suspension of resin. The finding that uniformly packed columns did not consistently result in uniform eluent rates, however, led to the taking of a second step. In this connection, it was found that the porosity of the Teflon sintered disc inserted into the fitting at the base of the column differed substantially from one disc to another. This finding led to the careful screening and selection of discs prior to their use.

The other factor that had to be reckoned with dealt with the volume and/or character of the buffer gradient. According to the operational instructions furnished with the analyzer[2], a volume of 75 ml was needed in each of the nine chambers of the Autograd for a single chromatographic run. For the three-column capability, this would necessitate the placement of 225 ml of buffer in each chamber. As the capacity of each chamber was approximately 160 ml, it was apparent that either an Autograd having at least three additional chambers must be used or a modification of the buffer gradient was needed. Fortunately, the buffer gradient described in our previous work[3] was found to elute satisfactorily the three columns when all of the nine chambers were filled to capacity. It was essential, however, to tilt the Autograd forward during the final 30 min of each run in order to utilize the entire volume.

ACKNOWLEDGEMENT

We are indebted to Mr. ALTON J. RAHE for statistical analyses. Research reported in this paper was conducted by personnel of the above named activity of the United States Air Force, at Brooks Air Force Base, Texas. Further reproduction is authorized to satisfy the needs of the U.S. Government.

REFERENCES

1 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
2 *Amino Acid Analyzer Instruction Manual AAA-1*, Technicon Corporation, Ardsley, N.Y., 1967.
3 J. P. ELLIS AND J. M. PRESCOTT, *J. Chromatogr.*, 43 (1969) 260.
4 H. MEYER, *Biochem. J.*, 67 (1957) 333.

CHROM. 5389

THE SEPARATION OF POLYCYCLIC AROMATIC HYDROCARBONS

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SUMMARY

A mixture of polycyclic aromatic hydrocarbons was separated in a column, packed with Al_2O_3 containing 2% by weight of water, by eluting with a gradient of cyclopentane–ether. By means of a flow-through-cell ultraviolet spectrophotometer, the extinctions of the eluted components were recorded at the wavelengths of 260, 275 and 296 nm at 30-sec intervals. Sensitivity and selectivity of the determination was thus considerably increased.

INTRODUCTION

As has been shown earlier[1], adsorption chromatography using a gradient of two solvents having different elution powers affords a number of advantages in group analyses of mixtures containing aromatic hydrocarbons with one to four rings. The time required for analysis is shortened and the eluates form sharp peaks which permit the detection of substances even though present in low concentrations. The earlier investigation[1] dealt with hydrocarbons containing four rings among which fluoranthene and pyrene were recorded separately and the others as a sum. There has been an ever growing interest in the analysis of polycondensed aromatic hydrocarbons, especially in connection with the carcinogenic properties that some of them have been found to posses. The recent stricter sanitary regulations and atmospheric purity control measures require new effective and sensitive analytical methods for the determination of these substances.

Most studies dealing with the determination of polycyclic aromatic hydrocarbons are based on preliminary separation by adsorption chromatography, using columns with Al_2O_3 adsorbent or silica gel, or by thin-layer chromatographic methods. After separation, the substance in question is determined in the fractions obtained, using UV spectrophotometry or low-temperature fluorescence. Separation on alumina-packed columns was used by CLEARY[2], GRIMMER AND HILDEBRANDT[3], SFORZOLINI *et al.*[4], who all subsequently measured the UV spectra of the individual fractions. Thin-layer chromatography was used by JANÁK AND KUBECOVÁ[5], HOOD AND WINEFORDNER[6], BIERNOTH[7], SCHAAD *et al*[8]. and MALÝ[9] to separate polycyclic aromatics.

The present report shows the application of gradient elution chromatography for analysing aromatic hydrocarbons comprising four and more nuclei. Separation is carried out in a column packed with alumina wetted with 2% w/w of water. The

solvent gradient formerly employed, pentane–ether, was replaced by cyclopentane–ether. Cyclopentane is a more polar solvent than pentane, has a low viscosity which is advantageous for chromatographic separation, and a low boiling point, which allows the easy concentration of the collected fractions. A steeper gradient was also used at the beginning of analysis since the method was being used for the determination of four and more cyclic aromatic hydrocarbons without regard to the more detailed separation of the lower components, *i.e.* aromatics with one to three nuclei. The UV spectrophotometric detection method which had been developed for this purpose allowed the selective determination of the components in question. Application of the method is demonstrated on a mixture of standards as well as on a black coal pitch extract.

EXPERIMENTAL

Materials

The details are given in our earlier work[1].

Alumina Woelm Eschwege Neutral with 2% H_2O was used as adsorbent. Before use the alumina was heated for 8 h at 400° and then deactivated by addition of 2% water.

A column of 4 mm inner diameter and 1 m in length, and a gradient elution pump (Dialagrad Model 190, ISCO) were used. The UV spectrophotometer was an SP 800 B (Pye Unicam). The spectrophotometer was connected to a three-channel point recorder allowing three-color records with 30-sec intervals between the points. The wavelength was changed at the beginning of each interval, and at its end the respective extinction value was recorded. Three records differing in color were thus obtained, each color corresponding to an extinction at one wavelength. For the purpose of increasing sensitivity, use was made of a flow-through quartz cell of 10 mm pathlength and 0.2 ml dead volume capacity.

Procedure

The method was verified with a mixture of standards containing fluoranthene, triphenylene, 1,2-benzanthracene, chrysene, 3,4-benzopyrene and coronene. 100 mg of a mixture of standards were dissolved in 1 ml 1-methylnaphthalene. The column was first wetted with pure cyclopentane and 1.5 μl of the standard mixture was then introduced. After injecting, the 2-h program of the gradient cyclopentane–ether elution was started. The flow rate of the eluents through the column was 60 ml/h. The detection of the eluted components at the wavelengths of 260, 275 and 296 nm is shown in Fig. 1 together with the gradient course.

Fig. 2 shows an example of analysis of a cyclohexane coal tar pitch extract. 5 g of black coal pitch was extracted in a Soxhlet with 200 ml cyclohexane for 10 h. The cyclohexane solution was evaporated *in vacuo* and 0.5 g of extract was dissolved in 1 ml benzene. 1.0 μl of benzene solution was dosed into the column. In the analysis of the black coal pitch the flow rate through the column and the gradient course were the same as in the case of separating the standard mixture. The fractions collected during analysis were subjected to UV spectral measurements. The individual eluates were identified by comparing the spectra with those of the standards. Fig. 3 shows the spectrum of a fraction containing 3,4-benzopyrene in comparison with a standard spectrum.

RESULTS AND DISCUSSION

The method reported earlier[1] in which the eluates were detected at 260 nm is not suitable for separating polycondensed aromatic hydrocarbons since at this wavelength some of these hydrocarbons do not provide a satisfactory response, and thus require considerably larger amounts of sample to be introduced which reduces the separating efficiency of the column. Simultaneous detection at several wavelengths was therefore chosen in order to ensure that most of the components are recorded in this manner. The earlier procedure, *i.e.* detection at a single wavelength, would require as many analyses as would correspond to the number of wavelengths selected for detection. Such a procedure would be very time consuming and any inaccuracy in the reproducibility of the experiments might cause serious errors. The present procedure makes use of the possibilities of flow-through cell UV spectrophotometry and the UV extinctions were recorded at three wavelengths at 30-sec intervals. Recording at the wavelengths of 260, 275 and 296 nm was proved suitable.

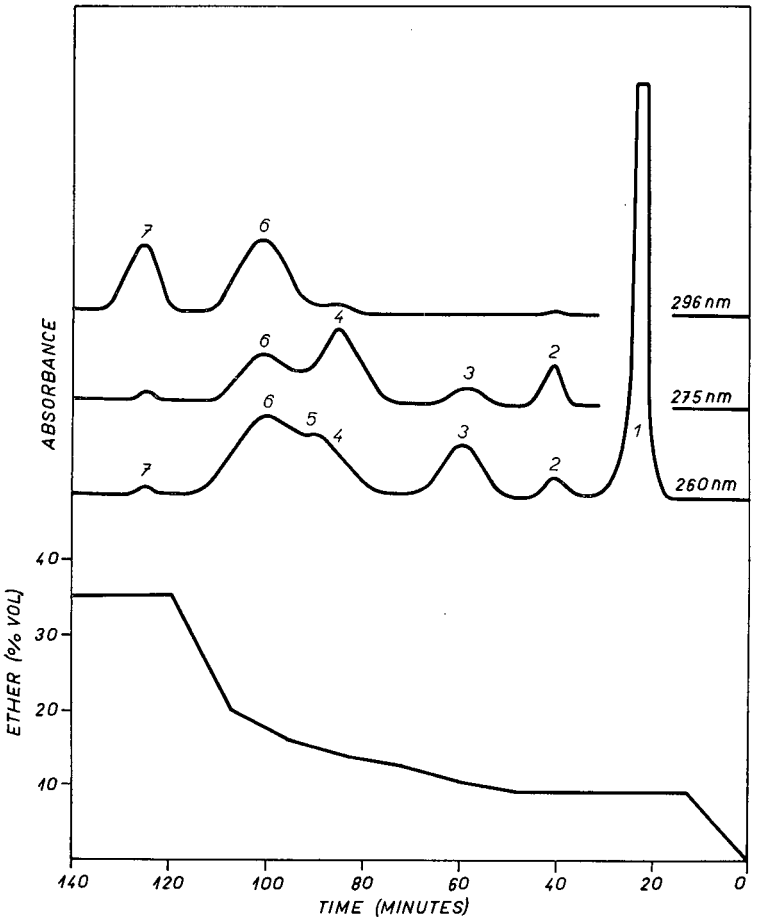

Fig. 1. Separation of the mixture of standards with recordings of the eluted compounds at three wavelengths. 1 = 1-methylnaphthalene (solvent); 2 = fluoranthene; 3 = triphenylene; 4 = 1,2-benzanthracene; 5 = chrysene; 6 = 3,4-benzopyrene; 7 = coronene.

Fig. 1, showing the separation of a mixture of standards, indicates an obviously satisfactory separation of 1-methyl naphthalene, fluoranthene, triphenylene, and coronene; the example of the threesome, 1,2-benzanthracene, chrysene and 3,4-benzopyrene also shows the advantage of simultaneous extinction recording at three different wavelengths. At 260 nm all the components remain virtually within a single extended peak. At 275 nm it is possible to distinguish clearly the peaks of 1,2-benzanthracene and 3,4-benzopyrene, while the extinction of chrysene is low and practically non-interfering at this wavelength. At 296 nm the extinction of 1,2-benzanthracene and chrysene is suppressed to a minimum and the record shows a sharp peak of 3,4-benzopyrene.

The method is sensitive, allowing the detection of 0.5 μg of 3,4-benzopyrene in the sample without amplification. When the quantity injected is known and the respective calibrations have been performed it is thus possible to determine quantitatively the content of 3,4-benzopyrene in a mixture.

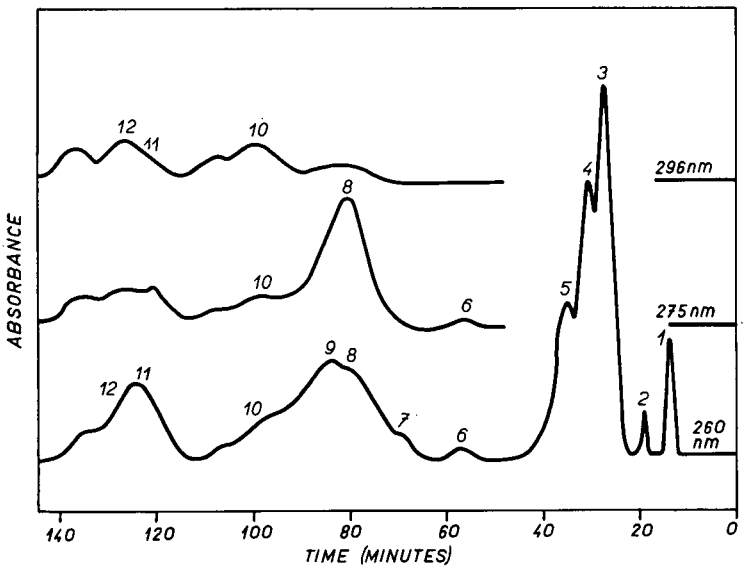

Fig. 2. Separation of coal tar pitch extract. 1 = benzene (solvent); 2 = two-nuclei hydrocarbons; 3 = phenanthrene, anthracene; 4 = pyrene; 5 = fluoranthene; 6 = triphenylene; 7 = benzofluorenes; 8 = 1,2-benzanthracene; 9 = chrysene; 10 = 3,4-benzopyrene; 11 = 1,12-benzoperyene; 12 = coronene.

As indicated by Fig. 2, which shows the analysis of a cyclohexane extract from black coal pitch recorded at 260 nm, the sample also contains aromatic hydrocarbons with two nuclei. Peak No. 1 pertains to benzene which was employed as solvent, peak No. 2 to a mixture of hydrocarbons with two rings. The further three partially separated components represent phenanthrene and anthracene (peak No. 3), pyrene and fluoranthene. Triphenylene is completely separated. Peak No. 7, pertaining to 1,2- and 2,3-benzofluorene, is quite small. Then further components follow in the order: 1,2-benzanthracene; chrysene and 3,4-benzopyrene, which at 260 nm constitute a single elongated peak. As shown by the UV spectrum of the 3,4-benzopyrene

fraction in Fig. 3, separation of this component from the other substances is very satisfactory and allows quantitative evaluation. The double peak following next was found to belong to 1,12-benzoperylene and coronene. At the 275 nm wavelength a well-defined peak of 1,2-benzanthracene and small peaks of triphenylene and 3,4-benzopyrene in addition to other less distinguishable components are detected. At 296 nm well discernible peaks of 3,4-benzopyrene and coronene appear as well as two further components which have not been identified. It should be noted that the substance analyzed as an example in Fig. 2 was neither treated nor purified in advance.

The reported method of gradient elution adsorption analysis of polycondensed aromatics, including simultaneous recording of the extinction at several wavelengths, has a number of advantages. In addition to the already mentioned possibility of detecting most of the eluted components, suitable selection of wavelength permits records of some eluates to be obtained either selectively with maximum suppression of interference from other substances or to achieve maximum sensitivity by choosing the wavelength so that the substance in question exhibits maximum extinction. Another advantage is afforded by the possibility of comparing the extinction records at three different wavelengths which may show whether a peak pertains to one or

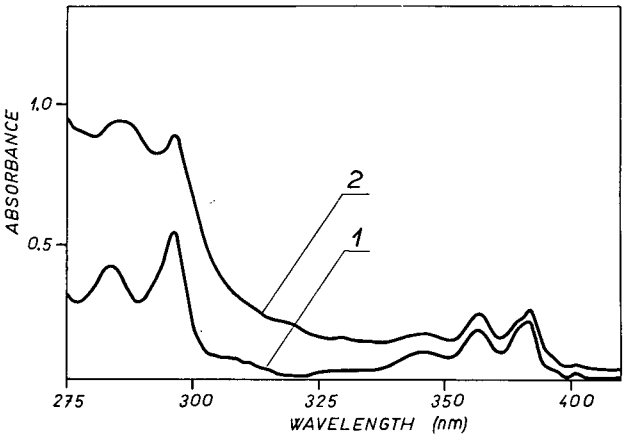

Fig. 3. UV spectrum of 3,4-benzopyrene. Curve 1 = standard; curve 2 = eluted fraction.

more components; in the case of known substances it may serve as evidence of their identity; it may also help in the identification of unknown substances. As shown in the example of 3,4-benzopyrene and 1,2-benzanthracene, this method allows the determination over the entire course of elution for a certain component which may then be collected and determined separately by spectrophotometry. In spite of the fact that the undesirable interference cannot be eliminated completely at any wavelength in such rich mixtures of substances, the method can nevertheless even serve for quantitative orientation determinations of certain substances.

For example, it is possible to prepare a calibration curve for 3,4-benzopyrene by plotting the values of the areas corresponding to the extinction of 3,4-benzopyrene at 296 nm *vs.* the quantity of 3,4-benzopyrene in the feed. When analysing an unknown mixture of polycondensed aromatic hydrocarbons the maximum amount of

3,4-benzopyrene in the feed is determined from the area corresponding to this substance. The course of extinction at two other wavelengths permits one to assess the possible interference of the other components. It is obviously necessary to maintain identical conditions of analysis as compared to those of the standards, especially as regards the flow rate of the eluent through the column.

The main advantage of the method is its speed, as the entire analysis takes only 2.5 h. The amount of sample required, which ranges from 0.1 to 0.5 mg, is likewise advantageous for determining polycyclic aromatic hydrocarbons in foodstuffs, in the atmosphere, etc. In addition to this the small quantity of sample required allows one to work within the linear adsorption isotherm region, this being significant from the standpoint of the reproducibility of results. No special treatment of the sample is necessary since saturated hydrocarbons and olefins are eluted at the beginning of the analysis and polar substances have much larger retention volumes than the aromatic hydrocarbons in question. It should be noted that hydrocarbons having a linear molecule, *e.g.* tetracene or pentacene, likewise have much higher elution volumes and are not eluted during the analysis described.

REFERENCES

1 M. POPL, J. MOSTECKÝ AND Z. HAVEL, *J. Chromatogr.*, 53 (1970) 233.
2 G. J. CLEARY, *J. Chromatogr.*, 9 (1962) 204.
3 G. GRIMMER AND A. HILDEBRANDT, *J. Chromatogr.*, 20 (1965) 89.
4 G. S. SFORZOLINI, F. PASCASIO, E. MARCHESOTTI AND M. N. CHIUCCHIU, *Ann. 1st. Super. Sanita*, 3 (1967) 45.
5 J. JANÁK AND V. KUBECOVÁ, *J. Chromatogr.*, 33 (1968) 132.
6 L. V. S. HOOD AND J. D. WINEFORDNER, *Anal. Chim. Acta*, 42 (1968) 199.
7 G. BIERNOTH, *J. Chromatogr.*, 36 (1968) 325.
8 R. SCHAAD, R. BACHMANN AND A. GILGEN, *J. Chromatogr.*, 41 (1969) 120.
9 E. MALÝ, *J. Chromatogr.*, 40 (1969) 190.

CHROM. 5399

THE INFLUENCE OF SOLVENT AND TEMPERATURE IN DEXTRAN GEL CHROMATOGRAPHY

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SUMMARY

The influence of solvent and temperature on the separations of oligosaccharides on dextran gels has been studied. Increasing temperature leads to an increasing partition coefficient, reflecting a greater activity of the solute in the gel phase when there are weaker gel–solvent interactions. Similarly, it was found that the better the solvent (the stronger the solvent–gel matrix interactions), the more the partition coefficient is changed to favour the mobile solvent phase. These results demonstrate the important part played by solubility-determined partitioning in separations on tightly cross-linked dextran gels.

INTRODUCTION

In ideal gel chromatography, the coefficient describing the partition between the mobile solvent phase and the gel phase is determined only by the change in configurational entropy corresponding to the free energy required to transport a solute molecule from the solvent to the gel phase. In polar systems, however, there may be interactions between the solute and gel phase characterized by an appreciable enthalpy term. In a previous communication[1], it was shown that in the case of tightly cross-linked dextran gels the separation is a function of both volume exclusion and partitioning determined by solubility behaviour, implying polar rather than steric interactions. The present investigation was undertaken to elucidate the relative magnitude of these effects as a function of temperature and solvent.

EXPERIMENTAL

The preparation of Sephadex G-15 columns was as previously described[2]. The dimensions are summarized in Table I. The non-aqueous solvents were reagent grade, dried over molecular sieves. The preparation and characterization of the cellodextrins and xylodextrins has been described elsewhere[1,2].

RESULTS AND DISCUSSION

Temperature dependence

In this section, the results are described in terms of K_{av} rather than K_D,

TABLE I

DIMENSIONS OF THE COLUMNS USED AT 25°

	DMSO	*Formamide*	*Water*	*0.1 M NaCl*
Settled bed volume (ml)	52.5	44.9	48.0	45.5
Void volume, V_0 (ml) (Blue Dextran)	26.4	19.8	19.4	19.0
Column height	66.9	57.2	61.1	58.0
Cross-sectional area (cm^2)	0.785			
Sample volume (ml)	0.1			
Sample concentration (mg/ml)	1			

as the former is more readily determined with precision when changes in the volume of the gel occur with variations in experimental conditions.

$$K_{av} = \frac{V_e - V_0}{V_T - V_0}; \qquad K_D = \frac{V_e - V_0}{V_I}$$

where V_T is the total volume and V_I the internal volume. For a discussion of these coefficients see, for example, ref. 3.

Fig. 1 shows that K_{av} increases with increasing temperature for the cellodextrins

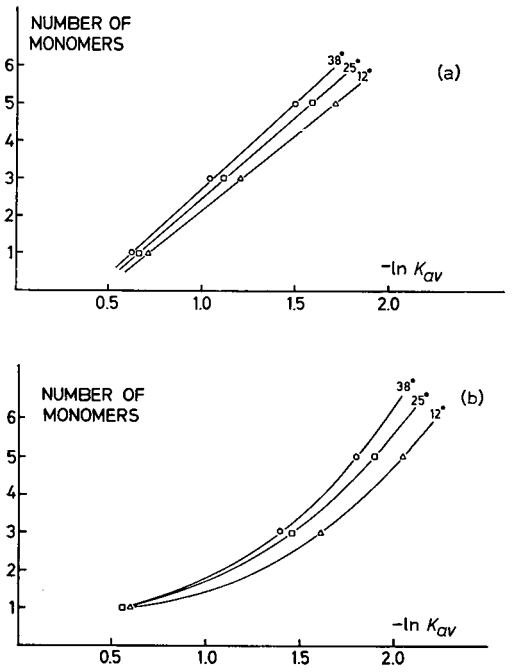

Fig. 1. Relationships at different temperatures between the number of chain units and $-\ln K_{av}$ for cellodextrins (a) and xylodextrins (b) on Sephadex G-15 (deionized water).

and xylodextrins on Sephadex G-15. Assuming equilibrium conditions, enthalpies may be determined from the dependence of ln K_{av} on temperature according to:

$$\Delta H^\circ = RT^2 \frac{\mathrm{d} \ln K_{av}}{\mathrm{d}T}$$

Free energies and entropies follow from:

$$\Delta G^\circ = -RT \ln K_{av}; \qquad \Delta S^\circ = \frac{(\Delta H^\circ - \Delta G^\circ)}{T}$$

The primary data are summarized in Table II. Fig. 2 shows the dependence of ln K_{av} on $1/T$. Values of ΔH°, ΔG° and ΔS° are given in Table III. With increasing temperature, the interactions between the solvent and the polysaccharide gel matrix decrease. This results in an increase in the activity of the solute in the gel phase (hence the positive sign of ΔH°) and consequently ΔH° will provide an index of the relative solubility behaviour of the solutes. The decreased interactions between

TABLE II

PARTITION COEFFICIENT, K_{av}, AS A FUNCTION OF TEMPERATURE FOR OLIGOSACCHARIDES ON SEPHADEX G-15 (DEIONIZED WATER)

	12°		25°		38°	
	K_{av}	$-\Delta ln\, K_{av}$	K_{av}	$-\Delta ln\, K_{av}$	K_{av}	$-\Delta n\, K_{av}$
Cellodextrin						
Glucose	0.48_8	0.48_7	0.51_3	0.44_8	0.53_0	0.40_9
Cellotriose	0.30_0	0.50_3	0.32_8	0.47_0	0.35_3	0.42_6
Cellopentaose	0.18_1		0.20_5		0.22_5	
$(V_T - V_0)$ (ml)	28.5		28.5		28.2	
Xylodextrin						
Xylose	0.54_8	1.0_0	0.56_8	0.89_5	0.56_8	0.83_6
Xylotriose	0.20_1	0.46_0	0.2_{33}	0.43_3	0.24_3	0.39_8
Xylopentaose	0.13_0		0.15_1		0.16_6	
$(V_T - V_0)$ (ml)	28.4		28.4		27.4	

TABLE III

ENTHALPY, FREE ENERGY AND ENTROPY PARAMETERS FOR OLIGOSACCHARIDES ON SEPHADEX G-15 AT 25° (DEIONIZED WATER)

	K_{av} (25°)	ΔH° ($cal \cdot mole^{-1}$)	ΔG° ($cal \cdot mole^{-1}$)	ΔS° ($cal \cdot mole^{-1}\ deg^{-1}$)
Cellodextrin				
Glucose	0.51_3	560	395	0.6
Cellotriose	0.32_8	1090	660	1.4
Cellopentaose	0.20_5	1450	940	1.7
Xylodextrin				
Xylose	0.56_8	265	335	−0.2
Xylotriose	0.23_3	1440	860	1.8
Xylopentaose	0.15_1	1660	1120	1.8

solvent and polysaccharides at higher temperature are evidenced by their well known large negative temperature coefficients of viscosity (due to decreased solvation) and their characteristic exothermic heats of dilution.

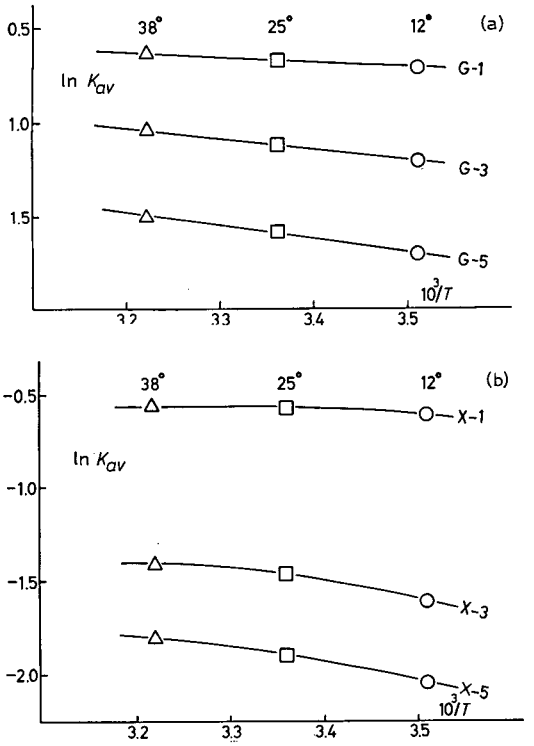

Fig. 2. Dependence of ln K_{av} on $1/T$ for cellodextrins (a) and xylodextrins (b) on Sephadex G-15 (deionized water). The increasing slopes suggest positive enthalpies increasing as the molecular weight increases.

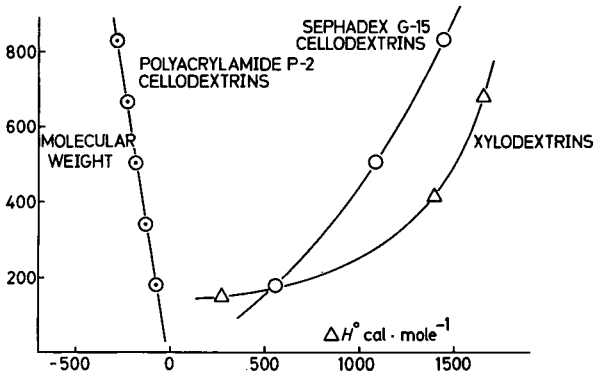

Fig. 3. Apparent enthalpies, reflecting the relative solubility behaviour of various oligosaccharides, as a function of molecular weight.

As previously noted[1], with dextran gels there is a solubility-determined partitioning of low-molecular-weight homologues between the gel and mobile solvent phases in addition to volume exclusion. The observed partition coefficient may thus be described as a function of $K_{\text{solubility partion}}$ and $K_{\text{volume exclusion}}$. As discussed below, the experimental evidence suggests that $K_{\text{volume exclusion}}$ is essentially independent of temperature for a given solute of the type considered here.

Fig. 3 shows that on Sephadex G-15, the relatively hydrophilic xylodextrins have a greater affinity for the solvent compared with the corresponding cellodextrins (with the exception of the monomers, as previously discussed[1]). The solubility behaviour of the oligosaccharides is not a simple function of their molar volumes, owing to the important part played by intramolecular hydrogen bonding. The extent of the latter may differ substantially for oligosaccharides of corresponding chain length but possessing only subtle differences in either conformation or structure. The upward curvature of the plots reflects the variation in solubility as a function of molecular weight. With polyacrylamide gels, the affinity of the solute for the gel increases linearly with molecular weight, suggesting an adsorption mechanism in this system[2].

Fig. 4 shows the correspondence between the separations with Sephadex G-15 and the relative solubility behaviour of the solutes. As would be expected, good correlation is also demonstrated in Fig. 5 between the separations in thin-layer chromatography (TLC) and $\Delta H°$.

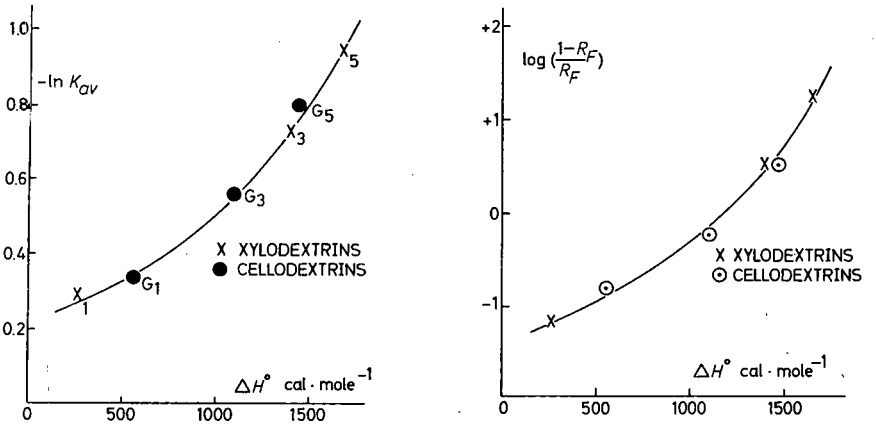

Fig. 4. Relationship between the separations of oligosaccharides on Sephadex G-15 and enthalpies reflecting their relative solubilities.

Fig. 5. Relationship between TLC data and enthalpies reflecting the relative solubility behaviour of various oligosaccharides.

It may be argued that the increasing partition coefficient with increasing temperature could be a consequence of the smaller hydrodynamic volume of the solute at the higher temperature. However, the partition coefficient shows a linear correspondence with the extended length of rigid rods, such as the oligosaccharides, in gel chromatography when solubility partitioning effects are absent[1]. Such behaviour has also been predicted by a statistical mechanical treatment[7]. The extended length of the cellodextrins is essentially invariant with temperature change[4] (at least up

to 70°). Furthermore, the observed partition coefficient does not correlate with the hydrodynamic volume of rod-like molecules in contrast to randomly coiling polymer chains. It may also be noted that the partition coefficient decreases with increasing temperature for the cellodextrins on polyacrylamide gels[2], whereas a smaller hydrodynamic volume would be expected to produce the opposite trend. Taken together, these observations suggest that $K_{\text{volume exclusion}}$ should exhibit little temperature dependence.

An alternative and more specific interpretation would be that the affinity of the less hydrophilic solutes for the dextran gel phase is due to adsorption. In this case, desolvation of the gel at the higher temperature promotes adsorption. Referring to Fig. 3, this would mean that the cellodextrins are more strongly adsorbed than the xylodextrins and that adsorption increases with molecular weight. The correlation of the TLC data with that from gel chromatography would simply mean that the tendency to adsorb is directly related to the decreasing solubility of the solute following Traube's law. Since both solubility partitioning and adsorption lead to a concentration of solute at the gel interface, the elucidation of such effects requires the use of an independent technique such as calorimetry. Preliminary micro-calorimetric results indicate a zero heat of mixing for cellopentaose, thus corresponding to solubility partitioning rather than adsorption since the accompanying heat of dilution is exothermic, reinforcing the heat of adsorption.

Solvent dependence

The dimensions of the Sephadex G-15 columns in some different solvents are given in Table I. As expected, the gel swells to an extent dependent on the polar nature of the medium (Table IV). The swelling is also reflected in differing void volumes (implying a deviation from spherical symmetry for the beads) which fall in the same order and are nearly in the same ratio as the swelling ratios given in the table.

TABLE IV

INTRINSIC VISCOSITIES OF GLUCOSE AND GEL SWELLING RATIOS IN DIFFERENT SOLVENTS

	$[\eta]_{25}$ $(ml \cdot g^{-1})$	*Gel swelling ratio*[a]	*Dipole moment, δ* $(cal \cdot g^{-1})$
DMSO	6.0_0	2.88	3.9
Formamide	4.0_0	2.62	3.25
Water[b]	2.68 (12°) 2.54 (25°) 2.36 (38°)	2.42	1.85
0.1 *M* NaCl	2.5_0	2.41	

[a] Ratio of swollen to dry volume.
[b] Interpolated from the results of IHNAT AND GORING[4].

Values of K_{av} are listed in Table V for the separations of the cellodextrins in the different solvents. Fig. 6 shows the relative magnitude of the solvent effect. The more pronounced the interactions between the solvent and the polymer constituting the gel matrix (falling in the order DMSO > formamide > water > 0.1 *M* NaCl), the lower is the activity of the solute in the gel phase, *i.e.* the smaller the partition coefficient.

TABLE V

PARTITION COEFFICIENT, K_{av}, FOR THE CELLODEXTRINS IN DIFFERENT SOLVENTS AT 25° (SEPHADEX G-15)

Cellodextrin	*Dimethyl sulphoxide*	*Formamide*	*Water*	*0.1 M NaCl*
G-1	0.341	0.500	0.513	0.528
G-2	0.190	0.376	0.415	0.427
G-3	0.104	0.293	0.328	0.336
G-4	0.058	0.213	0.259	0.272
G-5	0.025	0.144	0.205	0.215
G-6	Excluded	0.112	0.162	0.189

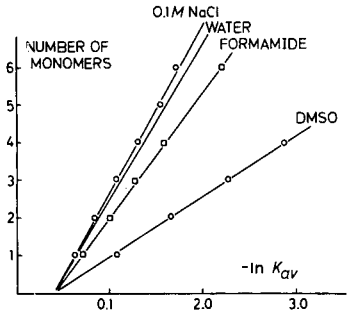

Fig. 6. Separations of the cellodextrins on Sephadex G-15 in different solvents.

Table IV lists intrinsic viscosities for glucose in DMSO, formamide and water and the dipole moments of these solvents. Since glucose may be approximated as a spherical molecule (axial ratio equals unity), the differences in intrinsic viscosity simply reflect the quantity of solvent bound to the molecule. The magnitude of the solute–solvent interactions thus increases in the order of the dipole moments of the latter and the swelling ratios for the gel matrix. However, the axial length of the solute is essentially independent of the degree of hydration[4]. Consequently, as in the case of temperature variation, $K_{\text{volume exclusion}}$ should be nearly independent of the solvent also, since the extended length is the parameter determining the elution volume in gel chromatography when solubility-partitioning effects are absent. The solvent effect may thus also be assigned to $K_{\text{solubility partition}}$, *i.e.* the better the solvent, the more the partition coefficient is changed in its favour relative to the gel phase. These results conform to the finding of ISHERWOOD AND JERMYN[8] that for the separation of oligosaccharides in TLC, the partition coefficient is a linear function of log N, where N is the mole fraction of water in the developing solvent.

The interpretation given above is necessarily qualitative and oversimplified. The real situation may well be more complex than visualized. For example, $K_{\text{volume exclusion}}$ may be expected to show some degree of solvent dependence, particularly with associated solvents such as DMSO and formamide. Nevertheless, this should not invalidate the conclusion that solubility partitioning is the major part of the temperature and solvent effects in tightly cross-linked dextran gels.

One may conclude that when the solutes to be separated have differing solubility behaviour as characterized by large excess heats of mixing (*i.e.* the interactions are polar in nature) solubility-determined partitioning will always occur together with partitioning controlled by volume exclusion. The extent to which solubility partitioning is important will depend on the magnitude of the solvent–gel matrix interactions.

Adsorption effects will occur when the solute or gel matrix possess functional groups which can interact specifically with each other. The latter forms a special case on the generally occurring partition phenomenon. As examples one may give the interactions of urea and formamide with dextran gels[5] and the adsorption phenomena encountered with oligosaccharides on cellulose gels[6]. In the latter, the primary alcohol groups apparently facilitate the adsorption of oligosaccharides. In contrast, the adsorption of such compounds to dextran gels apparently does not occur owing to the participation of this group in the main dextran structure (α-(1$\rightarrow$6) links).

ACKNOWLEDGEMENTS

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REFERENCES

1 W.BROWN AND Ö. ANDERSSON, *J. Chromatogr.*, 57 (1971) 255.
2 W. BROWN, *J. Chromatogr.*, 52 (1970) 273.
3 L. FISCHER, in *Laboratory Techniques in Biochemistry and Molecular Biology*, Interscience, New York, 1969.
4 M. IHNAT AND D. A. I. GORING, *Can. J. Chem.*, 45 (1967) 2363.
5 N. V. B. MARSDEN, *Ann. N.Y. Acad. Sci.*, 125 (1965) 478.
6 W. BROWN, to be published.
7 J. C. GIDDINGS, E. KUCERA, C. P. RUSSELL AND M. N. MYERS, *J. Phys. Chem.*, 72 (1968) 4397.
8 F. A. ISHERWOOD AND M. A. JERMYN, *Biochem. J.*, 48 (1951) 515.

CHROM. 5336

DISCONTINUOUS RECYCLING CHROMATOGRAPHY

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SUMMARY

A chromatography technique whereby the effluent is recycled in open one-column systems and which avoids the remixing of components of considerably different elution volumes is described.

INTRODUCTION

Chromatography using a recycling technique (recycling chromatography) was first introduced by PORATH AND BENNICH[1] in 1962, and represents a substantial improvement in those column chromatography systems in which the resolution is independent of the solute concentration, such as gel filtration. In this cyclic process, the effluent is pumped back to the influent end of the column in a closed circuit and the effective bed height can be increased several times. Since its introduction recycling chromatography has become a method of wide application[2].

If the process is carried out in an open system, as described in this paper, the effluent is collected in fractions, which after their evaluation, are reapplied to the column in the order of their emergence at an appropriately selected starting point in the elution profile. This discontinuous procedure, in which the column system is not connected in a closed circuit, permits the recycling, in one column, of mixtures of components with distribution coefficients ranging between 0 to 1 without their subsequent remixing. The number of cycles is not limited by the size of the column used. Recycling chromatography in an open system can be performed with conventional column systems without any additional equipment.

The procedure for discontinuous recycling chromatography is demonstrated in this paper by recycling on a Sephadex G-100 column of fragments from cyanogen bromide and trypsin hydrolysates of hog pepsin; the characterization of these components is the subject of further studies[3,4].

EXPERIMENTAL

Recycling of cyanogen bromide hydrolysate of hog pepsin

The discontinuous recycling procedure was carried out in a 260 × 10 cm bed of Sephadex G-100 equilibrated with a urea-containing eluant (0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0) which was also employed for the elution of the column.

The influent end (bottom) of the column was tapered to a thin tube, and was provided with a glass filter of medium pore size, 10 cm in diameter. The conical dead space below the filter was packed with glass beads, 2–3 mm in diameter. The effluent (top) part of the column was contracted to an internal diameter of 4 cm; it was closed off by another glass filter tapering to an outlet suitable for tubing of internal diameter approximately 1.2 mm (Fig. 1). This filter, which prevents the displacement of Sephadex particles from the column, was fitted in the bore of a rubber stopper fixed by a screw clamp into the neck of the column.

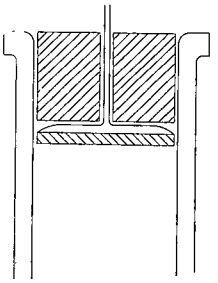

Fig. 1. Effluent part of the column. The chromatographic tube was shut off by a glass filter fitted into the bore of a rubber stopper.

The column was packed in the conventional manner, *i.e.* a slurry of the gel, prepared by allowing Sephadex G-100 (40–120 μ) to swell for 72 h in the urea-containing eluant described above, was allowed to sediment by gravity in the tube. The outlet end of the tubing connected to the bottom part of the column was adjusted so that the vertical distance between the level in the column and the outlet end of the tube did not exceed 1 m. After the packing had been almost completed, the column was closed by insertion of the rubber stopper with the outlet filter. The urea-containing eluant was then passed upward through the bed at a flow rate of 100 ml/h using a piston pump (Micropump MC 300, Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague). During this step the gel layer filled up the small dead space around the filter at the effluent end. The inlet tubing of the micropump, dipped into the urea-containing eluant in a reservoir, was provided with an inlet filter preventing the entry of impurities into the system.

The chromatography was effected in the upward flow arrangement. The inlet filter was immersed in the sample solution and transferred to the eluant reservoir after application of the sample to the column. The effluent emerging from the column passed through an ultraviolet absorptiometer (8300 Uvicord II, LKB Producter AB, Stockholm, Sweden), set at 280 nm, and then to a fraction collector. Fractions (100 ml) were collected at 1-h intervals and their absorbance at 280 nm was measured. The absorptiometer record was used to obtain instant information of the existent stage of separation in the column.

The preparation and purification of S-sulfo-pepsin from commercial twice crystallized hog pepsin (Worthington, Freehold, N.J., U.S.A.) as well as its cleavage with cyanogen bromide[5] were carried out as described elsewhere[6]. For the fractionation, 6.2 g of the cyanogen bromide hydrolysate was dissolved in 200 ml of the

urea-containing eluant (see above) whose pH had been adjusted to 8.5 by ammonium hydroxide. After the sample had dissolved completely, glacial acetic acid was added to decrease the pH of the sample to 5.0, and the sample solution was pumped into the column. The course of the fractionation is shown in Fig. 2. When fraction No. 170 was emerging from the column (as indicated by an arrow) reapplication of fractions Nos. 52–165 was begun; these fractions account for the entire effluent area corresponding to peaks CB1 – CB5. The inlet filter was placed at the bottom of a 100-ml measuring cylinder into which the first fraction (No. 52) to be recycled had been poured. After this fraction had been pumped almost completely into the column (avoiding the entry of air into the filter), fraction No. 53 was poured into the cylinder. The reapplication was continued in this manner up to fraction No. 165. The inlet filter was then transferred to the reservoir of eluant with which the development of the column was completed.

In the second cycle, during which the effective bed height amounted to 520 cm, the fractionation of the components present in the sample was substantially improved. Fractions corresponding to the individual peaks CB1 – CB5 were pooled, desalted on a Sephadex G-25 column, lyophilized, and investigated further[3].

Recycling of tryptic digest of an aminoethylated fragment CB2

The cyanogen bromide fragment of S-sulfo-pepsin was obtained as described above. It represents the N-terminal portion of the pepsin molecule and contains approximately 150 amino acid residues[4], among their number the only histidine of pepsin[7] and two half-cystine residues which form a disulfide loop in the molecule of the native enzyme ... –Cys–Ser–Ser–Leu–Ala–Cys–Ser–Asp–His–... (refs. 8, 9). This fragment does not contain arginine or lysine residues[3]. The conversion of fragment CB2 into its aminoethyl derivative[10], the digestion of the latter with trypsin, and the characterization of the individual tryptic fragments will be described in detail in a forthcoming paper[4].

The cleavage of the aminoethylated fragment CB2 at the carboxyl side of the two S-(β-aminoethyl)-cysteinyl (AEC) residues gave rise to a pentapeptide, Ser–

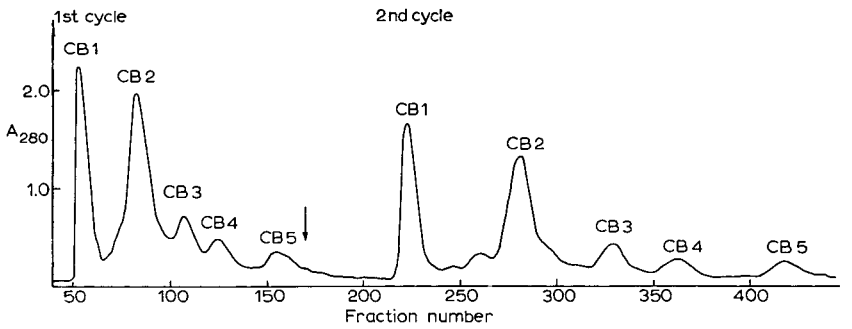

Fig. 2. Discontinuous recycling chromatography of the cyanogen bromide hydrolysate of S-sulfo-pepsin. The fractionation of 6.2 g of sample dissolved in 200 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0, was effected on a Sephadex G-100 column (260 × 10 cm) equilibrated and eluted with the same solution. 100 ml fractions were collected at 1-h intervals. When fraction No. 170 was emerging (as marked by an arrow) reapplication of fractions No. 52–165 to the column was begun.

Ser–Leu–Ala–AEC, derived from the area between the AEC-residues, and to two larger fragments representing the rest of the molecule of fragment CB2. The pentapeptide was separated by gel filtration on a Sephadex G-25 column from the high molecular weight portion of the digest (designated CB2–RAE–T1). The latter was subjected to discontinuous recycling chromatography on the Sephadex G-100 column. The fractionation of this material is adduced here as another example of the applications of the fractionation technique described.

For the fractionation, 2.5 g of CB2–RAE–T1 was dissolved in 500 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0. The fractionation was carried out with the same equipment and under conditions identical to those described above for the fractionation of the cyanogen bromide hydrolysate. As is apparent from Fig. 3, the recycling was only aimed, in this case, at the isolation of the two main components, designated by the abbreviated symbols –T11b and –T12. After four cycles the effective bed height amounted to 10.4 m.

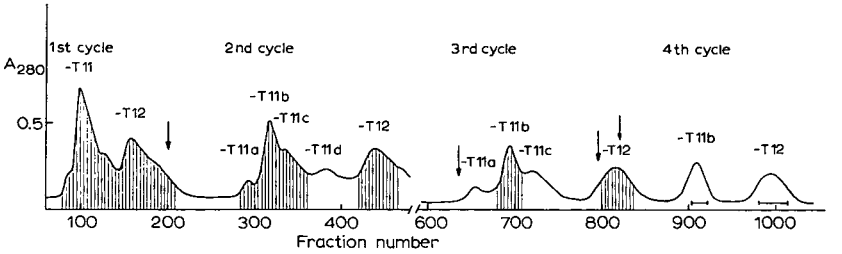

Fig. 3. Discontinuous recycling chromatography of tryptic digest of aminoethylated fragment of pepsin, CB2. The sample (2.5 g) was dissolved in 500 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0. The column and the conditions of the fractionation were identical to those described in the legend to Fig. 2. The areas selected for recycling are hatched. The sites of reapplication of the fractions to be recycled are marked by arrows, save for the application of areas –T11a through –T11c in the third cycle, which is at fraction No. 560 lying in the interrupted part of the diagram. The arrow at fraction No. 637 designates the beginning of the reapplication of area –T12.

RESULTS AND DISCUSSION

Exceptionally long beds are required to achieve good resolution of certain components by gel filtration. The height of the columns used is limited by various factors, such as, *e.g.* the mechanical properties of the gels, the required quantity of the bed material, etc. Recycling chromatography, first introduced by PORATH AND BENNICH[1], permits the effective bed height of smaller columns to be increased by pumping the effluent back to the influent end of the column. The fractionation of the components present is thus continued during their subsequent passage through the column. These cycles can be repeated several times and the course of the separation can be monitored by a flow analyzer connected into the circuit. It is important that the dead space of the system, where remixing takes place, be kept at a minimum. A commercial apparatus designed for chromatographic separations by recycling is available (4900A ReCyChrom, LKB Produkter AB, Stockholm, Sweden).

The recycling chromatography arrangement described, however, is subject to certain limitations. In the case where the mixture to be separated contains components with considerably different distribution coefficients (K_D), then a component charac-

terized by a low K_D value, which emerges from the column in a small elution volume, can after reapplication and passage through the column get mixed with some other, "slower" component with a higher K_D value. The possibility of this remixing increases, especially in the subsequent cycles due to the broadening of the zones. This effect together with the column size are factors which determine the number of cycles possible. These drawbacks can be circumvented by limiting the recycling to components with K_D values distributed over a relatively narrow range. The number and the distribution of the components and the program of recycling are determined in an exploratory experiment. A selector valve, connected in the circuit, permits the reapplication of a narrower effluent range, followed by the opening of the closed circuit, and the "bleeding out" of the remaining peaks to a fraction collector. In an alternative procedure[11], the selected component is transferred to another column in which its recycling is effected.

In the discontinuous procedure, the circuit is open and the effluent is collected in fractions. After their evaluation, the fractions to be recycled are reapplied to the column at an appropriately chosen point of the elution process and the circuit is thus again closed.

There is an essential difference between mere rechromatography and discontinuous recycling chromatography. In the first case the fractions selected for rechromatography are pooled and all the components present are mixed in the reapplied sample and enter the column as a homogeneous mixture. In the second case the fractions to be recycled enter the column in sequence and the mutual shift of zones, arising from the preceding separation step, is thus retained. The interruption of the circuit offers the possibility of a more complete evaluation of the effluent between the cycles, including the determination of enzymatic activity.

If the recycled region includes all the peaks (Fig. 2), then the reapplication is usually started after the first cycle has been completed. If the procedure is merely aimed at the isolation of some of the components, only selected peaks are recycled (Fig. 3). The recycled peaks are applied to the column in the order of their increasing elution volumes, *i.e.* in order of their emergence from the column. For a better distribution of peaks in the effluent, a volume of eluant can be inserted between individual recycled regions. At the beginning of each successive cycle the sequence of the increasing elution volumes is broken, *i.e.* the last peak of the largest elution volume is followed again by the first, "fastest" component. Reapplication can be begun after the "slowest" component has emerged or, alternatively, the point of application has to be determined by a comparison of the elution volumes of both components as determined in the first cycle. Under these conditions, components with K_D values distributed over the entire range, from 0 to 1, can be recycled in one single column.

The broadening of peaks as a result of diffusion is relatively low. No irregularities in the elution profile due to the discontinuity in the applied samples were observed when the volume of the fractions collected was small. Thanks to the upward flow arrangement, the flow resistance of the 260 cm tall bed of Sephadex G-100 did not increase even after several months of operation. The discontinuous procedure has also been used in the downward flow arrangement and with considerably smaller columns[12]. A factor of crucial importance is the keeping of the dead space at a minimum.

A certain drawback of the discontinuous procedure represents the time-consuming application of samples, which can be controlled automatically, however, at the cost of the simplicity of the equipment.

The procedure described can be used in combination with the continuous method. By recycling in an open system a degree of resolution can be obtained which allows cuts to be made unambiguously. The latter can then be subjected to final separation by the continuous procedure.

REFERENCES

1 J. PORATH AND H. BENNICH, *Arch. Biochem. Suppl.*, 1 (1962) 152.
2 *Literature references*, Pharmacia Fine Chemicals AB, Stockholm, Sweden.
3 L. MORÁVEK, V. KOSTKA AND F. ŠORM, in preparation.
4 L. MORÁVEK, V. KOSTKA AND F. ŠORM, in preparation.
5 E. GROSS AND B. WITKOP, *J. Biol. Chem.*, 237 (1962) 1856.
6 V. KOSTKA, L. MORÁVEK, I. KLUH AND B. KEIL, *Biochim. Biophys. Acta*, 175 (1969) 459.
7 T. G. RAJAGOPALAN, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 241 (1966) 1190.
8 J. TANG AND B. S. HARTLEY, *Biochem. J.*, 118 (1970) 611.
9 B. KEIL, L. MORÁVEK AND F. ŠORM, *Collect. Czech. Chem. Commun.*, 32 (1967) 1968.
10 M. A. RAFTERY AND R. D. COLE, *J. Biol. Chem.*, 241 (1966) 3457.
11 A. CHERSI, *Sci. Tools*, 11 (1964) 1.
12 V. KOSTKA, L. MORÁVEK AND F. ŠORM, unpublished results.

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STUDIES ON THE MECHANISM OF ADSORPTION OF PURINES IN SEPHADEX G-10 CHROMATOGRAPHY

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SUMMARY

The adsorption of purines to Sephadex G-10, as measured by the logarithms of corrected chromatographic elution volumes, V_e^0, shows correlations with the following physical properties of the purines: π-electron delocalization energy, electron donating ability, electron accepting ability, basic and acidic pK_a and water solubility. These correlations, together with equilibrium dialysis studies of adsorption of purines to dextran and Sephadex, indicate that purines interact primarily with the dextran portion of Sephadex in contrast to substituted benzene derivatives, which interact primarily with the ether cross-linkages of Sephadex.

INTRODUCTION

The Sephadex gels (Pharmacia) are composed of dextran, a linear $\alpha(1\rightarrow 6)$ glucose polymer with $\alpha(1\rightarrow 3)$ branching points, which has been cross-linked with epichlorohydrin to give glyceryl ether linkages of the type: dextran–O–CH_2–CH(OH)–CH_2–O–dextran[1]. Originally employed as supports for zone electrophoresis and as gel filtration columns for determination of molecular weights of macromolecules[1], Sephadex gels were shown by GELOTTE[2] to adsorb reversibly aromatic and heterocyclic compounds. The highly cross-linked Sephadex G-10 has been shown to be a useful adsorbent for column chromatography of purines and related compounds[3–9]. The effects of extent of gel cross-linkage[8,10,11] and eluent flow rate[7], ionic strength[7,11] and pH[6,7,11–13] have been studied in order to improve the separation of purines by Sephadex chromatography. However, the specific chemical groups of the purines and the Sephadex gels involved in adsorption and the types of bonds formed have not been determined. Investigations of the mechanism of adsorption of other heterocyclic and aromatic compounds to Sephadex indicated some relationship between substituent group properties such as increasing electron donating ability and increasing elution volumes on Sephadex columns[14,15]. BROOK

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and coworkers showed that the adsorption of substituted phenols[16] and substituted anilines and benzoic acids[17] correlated with the electron donating ability of the substituent groups as measured by their Hammett σ values and therefore that adsorption was due to the hydrogen bonding of the amino, carboxyl, or hydroxyl group to the gel cross-linkages. DETERMANN AND WALTER[18] showed by equilibrium dialysis that the adsorption of phenol to Sephadex gels was due to interaction with the ether cross-linking groups and not the dextran portion of the gel.

The availability of data for Sephadex G-10 chromatography at pH 7.0 of a variety of substituted purines and related compounds previously reported[7], together with the additional data given here, suggested that studies of the correlations of elution parameters with structural and physicochemical properties of the purines could give considerable information about the mechanism of adsorption of purines to Sephadex gels. Further studies with different dextran gels were performed to determine whether the ether linkages of Sephadex are involved in the adsorption of purines as they are in the adsorption of benzene derivatives.

METHODS

The selection of suitable conditions for Sephadex G-10 chromatography of purines and the methods used have been described[7]. All compounds were chromatographed on a 1.0 × 100 cm column of Sephadex G-10 eluted with 0.05 *M* sodium phosphate buffer, pH 7.0. In addition, some purines and heterocyclic compounds were chromatographed on a 1.0 × 50 cm column of Sephadex LH-20 (hydroxypropylated Sephadex G-25) eluted with the same buffer. The void volumes (V_0) were determined as the elution volumes (V_e) of dyed blue dextran and the internal volumes (V_i) were calculated from the elution volumes of acetone minus the void volumes. The corrected elution volumes (V_e^0) of the compounds were calculated from $V_e^0 = (V_e - V_0)/V_0$ (ref. 7).

Equilibrium dialysis experiments were performed with solutions of Dextran T 40 (mol. wt. 40,000) and suspensions of Sephadex G-10 (water regain 1.0, 40–120 μ), obtained from Pharmacia, at concentrations between 0 and 35% (w/w) in 0.05 *M* sodium phosphate, pH 7.0. 3 ml of these solutions or suspensions in dialysis tubing were dialyzed against 6 ml of the phosphate buffer, or 0.1 m*M* adenine or 0.1 mmol xanthine in the same buffer. Equilibrium was reached in 4–5 h. The change in absorbance at 260 nm between 0 and 6 h for the adenine and xanthine dialysis solutions, corrected for the change in absorbance of buffer dialysis solution, was used to calculate the per cent change in purine concentration as a function of per cent of Dextran or Sephadex in the dialysis tubing.

Regression equations and correlation coefficients were calculated using programs supplied with the Olivetti-Underwood Programma 101.

RESULTS

The advantage of using V_e^0 as a measure of adsorption instead of K_d, defined as $(V_e - V_0)/V_i$ (ref. 1), where V_i was calculated from the difference between the elution volumes of dextran and acetone, is shown in Table I. Columns were prepared from two lots of Sephadex G-10 and a standard solution of four purines was chroma-

TABLE I

COMPARISON OF ELUTION PARAMETERS FOR PURINES CHROMATOGRAPHED ON 1.0 × [illegible] cm COLUMNS FROM TWO LOTS OF SEPHADEX G-10

Compound	*Lot 1*			*Lot 2*		
	V_e (*ml*)	K_d	V_e^0	V_e (*ml*)	K_d	V_e^0
Dextran	32.5			31.2		
Acetone	61.0			56.1		
Hypoxanthine	113.0	2.82	2.48	104	2.92	2.34
Xanthine	156	4.33	3.59	143	4.49	3.59
Guanine	199	5.84	4.85	181	6.02	4.81
Adenine	251	7.67	6.38	231	8.02	6.40

TABLE II

ELUTION VOLUMES OF PURINES AND RELATED COMPOUNDS ON SEPHADEX G-10

Compound	V_e (*ml*)	V_e^0	*Log* V_e^0
Purines			
Guanine(-3-)N-oxide	91	1.70	+0.231
8-Methyltheophylline	105	2.24	+0.352
3-Methylxanthine	125	2.72	+0.436
1,3-Dimethyluric acid	131	3.03	+0.482
3-Methyladenine	138	3.25	+0.512
1,7-Dimethyluric acid	145	3.46	+0.540
7-Methyluric acid	147	3.52	+0.547
1-Methyluric acid	170	4.23	+0.627
8-Mercaptopurine	329	9.15	+0.963
6-Selenopurine	347	9.68	+0.986
8-Nitrotheophylline	437	12.01	+1.081
8-Chloroxanthine	601	17.05	+1.232
6-Piperidino(1)purine	606	17.31	+1.240
6-Benzylaminopurine	1413	42.48	+1.628
Ribosyl purines			
N^2-Dimethylguanosine	92	1.83	+0.263
Ribosyl 6-mercaptopurine	138	3.14	+0.497
Ribosyl 6-methylmercaptopurine	177	4.33	+0.637
Isopropylideneadenosine	199	4.99	+0.698
8-Azapurines			
8-Azatheophylline	188	4.80	+0.682
Pyrazolo[3,4-*d*]pyrimidines			
4-Amino-6-hydroxy-	175	4.29	+0.632
Pyrimidines			
5-Acetylamino-6-amino-1,3-dimethyluracil	55	0.70	−0.151
5,6-Diamino-1,3-dimethyluracil	80	1.46	+0.166
Miscellaneous			
NADP	34	0.037	−1.432
Pyridoxal phosphate	45	0.38	−0.414
Trigonelline	46	0.41	−0.387
Nicotinic acid	82	1.53	+0.168
N-Methyl-2-pyridone-5-carboxamide	88	1.71	+0.233
Urocanic acid	102	2.11	+0.325
Benzoic acid	111	2.34	+0.371
Nicotinamide	112	2.46	+0.391
Ribosyl-5-amino-4-imidazolecarboxamide	117	2.50	+0.398

tographed on each column. The mean of the differences in K_d for the two lots of Sephadex G-10 was 0.20 ± 0.05 (S.E.) which gave a p value of 0.045 when compared by Student's t-test. The mean of the differences for V_e^0 was only 0.04 ± 0.04 indicating no significant difference in V_e^0 for the two lots.

V_e, V_e^0 and log V_e^0 values for 91 compounds chromatographed on Sephadex G-10 columns have been published[7] and data for 32 additional compounds are presented in Table II. It should be noted that log V_e^0 for non-adsorbed compounds with $V_e = V_0 + V_i$ is —0.056. Substituent Δlog V_e^0 values are defined as the change in log V_e^0 due to the addition of one group on the purine ring, with a positive value indicating increased adsorption and a negative value indicating decreased adsorption[7]. Additional Δlog V_e^0 values for purine ring substituents are given in Table III. The additivity of Δlog V_e^0 values and their use in predicting elution volumes has been described[7].

Table IV lists substituent Δlog V_e^0 values for various ring structures related to the purine ring, with the ring positions numbered similarly. The change in adsorption for a given substituent is in the same direction for the three-ring systems, with the values similar for the purine and pyrazolo[3,4-*d*]pyrimidine rings but not

TABLE III

Δlog V_e^0 VALUES FOR SUBSTITUENTS ON THE PURINE RING

	Group	*Parent compound*	Δlog V_e^0
N_1	Methyl	3-Methylxanthine	—0.026
		7-Methyluric acid	+0.007
		Uric acid	+0.015
C_2	Hydroxyl	1-Methylhypoxanthine	+0.244
	Amino	1-Methylhypoxanthine	+0.323
		6,8-Dihydroxypurine	+0.330
N_3	Methyl	Xanthine	—0.119
		Adenine	—0.293[a]
		1-Methyluric acid	—0.145
	N-Oxide	Guanine	—0.455[a]
C_6	Hydroxyl	2-Hydroxypurine	+0.372
	Seleno	Purine	+0.986
	Benzylamino	Purine	+1.302
	Piperidino(1)	Purine	+1.240
N_7	Methyl	1-Methyluric acid	—0.087
		Uric acid	—0.135
		3-Methylxanthine	—0.182
C_8	Hydroxyl	Theophylline	+0.072
		1-Methylxanthine	+0.137
		7-Methylxanthine	+0.141
		1,7-Dimethylxanthine	+0.181
	Chloro	Xanthine	+0.677
	Nitro	Theophylline	+0.671
	Mercapto	Purine	+0.963
	Methyl	Theophylline	—0.058
N_9	Ribosyl	N^2-Dimethylguanine	—0.273
		6-Methylmercaptopurine	—0.309
		6-Mercaptopurine	—0.354
	Isopropylidene-ribosyl	Adenine	—0.107

[a] Introduces ⊕ in ring.

for the 8-azapurine ring. Also shown in Table IV is the major exception to the rule that substituent $\Delta\log V_e^0$ values are independent and additive. The value for a hydroxyl group at any position on the rings is positive except for the addition of a hydroxyl group at position 2 of a ring with a hydrogen or amino group at position 6. The ring $\Delta\log V_e^0$ values in Table IV indicate that with the exception of 2,6-diamino-8-azapurine, the ring $\Delta\log V_e^0$ values are largely independent of the substituent groups on the rings. Removal of the imidazole portion of the purine ring greatly reduces adsorption as shown by the large negative purine to pyrimidine ring $\Delta\log V_e^0$ of —0.298. Conversion of the imidazole ring of purine to a triazine ring (mean $\Delta\log V_e^0 = +0.229$) or to a pyrazolo ring (mean $\Delta\log V_e^0 = +0.179$) greatly increases adsorption. The ring $\Delta\log V_e^0$ value of +0.319 for the change from imidazole to pyrazole is 1.8 times the value for conversion of purine to pyrazolo[3,4-*d*]-pyrimidine, indicating that the pyrimidine ring of these compounds influences the effects of changes in the five-membered ring.

The effects of substituents on the purine ring on $\log V_e^0$ values suggest that a high electron density in the ring favors adsorption to Sephadex. Uric acid and methylated uric acids which exist as anions at pH 7.0 are strongly adsorbed while those compounds that contain a positive charge due to quaternization of a ring

TABLE IV

$\Delta\log V_e^0$ VALUES FOR VARIOUS RING SYSTEMS NUMBERED ANALOGOUS TO THE PURINE RING

2-Amino group substituent $\Delta\log V_e^0$ values

Parent compound	*Purine*	*8-Azapurine*
6-Amino	+0.293	+0.021
6-Hydroxyl	+0.320	+0.163

2-Hydroxyl group substituent $\Delta\log V_e^0$ values

Parent compound	*Purine*	*8-Azapurine*	*Pyrazolo[3,4-d] pyrimidine*
6-Amino	—0.304	—	—0.380
6-Hydroxyl	+0.189	+0.232	+0.162

Ring $\Delta\log V_e^0$ values

Parent purine ring to	*Pyrimidine*	*8-Azapurine*	*Pyrazolo[3,4-d] pyrimidine*
2,6-Diaminopurine	—	+0.001	—
Guanine	—	+0.156	—
Theophylline	—	+0.272	—
Adenine	—	+0.273	+0.207
Hypoxanthine	—	+0.313	+0.203
Xanthine	—0.333	+0.357	+0.176
Isoguanine	—0.294	—	+0.131
Purine	—0.266	—	—
Mean value	—0.298	+0.228	+0.179
S.E.M.	0.019	0.053	0.017

nitrogen or an amino group have greatly reduced adsorptions. Also, the purines in the lactim form, which are more aromatic, are more strongly adsorbed than are the purines in the lactam form. This is shown by the log V_e^0 values of +0.366 for 6-hydroxypurine (lactam) compared to +0.556 for 6-methoxypurine (lactim) and also +0.851 for 6-mercaptopurine (thione) compared to +0.946 for 6-methylmercaptopurine (mercapto). This increased adsorption is not due to the methyl group on the sulfur or oxygen substituent since the log V_e^0 value for 6-methylaminopurine of +0.826 is only slightly higher than that of +0.805 for 6-aminopurine.

The ring nitrogens or the hydrogen groups on the ring nitrogens of purines in the lactam form are of major importance for adsorption to Sephadex as shown by the large decrease in adsorption when the hydrogen is replaced by a methyl group (Table V). Methyl substitution at the 1 position gives the smallest Δlog V_e^0 value and hence this position contributes little to the adsorption of the purine ring. The values for a methyl group at positions 3, 7 or 9 are comparable indicating that these positions contribute about equally to the adsorption. This decrease in adsorption upon methyl substitution on the ring nitrogens is not a simple steric effect since a methyl group at the 6 carbon has a small positive Δlog V_e^0 value while a methyl group at the 8 carbon has a small negative Δlog V_e^0 value. Assuming that the negative of the methyl Δlog V_e^0 values is a measure of the contribution of the ring nitrogens to adsorption of the purine ring, the negative of the sum of the mean Δlog V_e^0 values for positions 1, 3, 7 and 9, +0.404, when added to the value for a nonadsorbed compound (—0.056) gives +0.348. This value can account for the log V_e^0 value of +0.326 observed for purine.

To obtain further information about the mechanism of adsorption of purines to Sephadex G-10, correlations of elution parameters with the physical properties of the purines were investigated. The appropriate elution parameter is log V_e^0 which is proportional to the logarithm of an adsorption coefficient which in turn is proportional to the difference in standard chemical potential between the molecules in solution

TABLE V

Δlog V_e^0 VALUES FOR METHYL GROUPS ON THE PURINE RING

	Ring position						
	N_1	N_3	N_7	N_9	N^{+a}	C_6	C_8
Mean	—0.048	—0.150	—0.134	—0.172	—0.457	+0.084	—0.058
S.D.	0.044	0.020	0.026	—	0.195	—	—
S.E.M.	0.015	0.009	0.008	—	0.098	—	—
n	8	5	9	1	4	1	1

	Ring position	
	N_6 (*amino*)	N_2 (*amino*)
First methyl	+0.021	—0.041
Second methyl	+0.058	—0.109
Third methyl	—0.326[a]	—

[a] Methyl group causes quaternization of amino group or ring nitrogen.

and those adsorbed to Sephadex. Thus, the following equation relates log V_e^0 and chemical potential:

$$\log V_e^0 = \frac{\mu^0_{liq} - \mu^0_{gel}}{2.33\,RT} + C$$

where μ^0 refers to the standard chemical potentials, R is the gas constant, T is the absolute temperature, and C is a proportionality constant. Since the values of log V_e^0 were determined at the same temperature (room temperature), log V_e^0 increases as the standard chemical potential of the compound on the gel is reduced relative to that of the solution. Differences in the standard chemical potentials of purines are related to their physical properties and therefore correlations between physical properties and log V_e^0 values are expected.

The correlations of the physical properties of the purines with their log V_e^0 values to be described concern mainly methyl, amino, and hydroxyl substituted purines since it appears that these groups exert their effects primarily through changes in the properties of the rings. Purines containing sulfur, halogen, carboxyl and cyano groups consistently failed to show correlations between their physical properties and adsorption to Sephadex. This is probably due to direct interaction of these groups with the gel and therefore these compounds were excluded from the correlations except where noted. It is interesting that the molecular weights of the halogens correlate well with their Δlog V_e^0 values for the 6 position of the purine ring (Fig. 1), and a similar trend is found for the chalcogens. Polarizability of the halogens is proportional to their molecular weights while electronegativity is inversely proportional. Therefore, adsorption of the halogen groups to Sephadex may involve dipole-induced dipole and dispersion forces. BROOK AND MUNDAY[17] have noted that halogen-substituted phenols, anilines and benzoic acids are more strongly adsorbed to Sephadex than predicted by their Hammett σ relations, and that the order of increasing adsorption was F, Cl, Br, I.

The greater adsorption of purines with the lactim structure compared to those with the lactam structure suggested that increased aromatic character might correlate with adsorption to Sephadex. Although few experimental data are available on the electronic structure of purines, an extensive series of molecular orbital (MO) calculations which give approximate values for the electronic properties of purines have been published by the PULLMANS[19,20]. The values used for the correlations with log V_e^0 values were those calculated by the Hückel MO Linear Combination of Atomic Orbitals method, with the imidazole hydrogen assumed to be at the 9 position.

The delocalization energy, E, a measure of the aromatic character of the compounds, was found to give a fair correlation with log V_e^0 for a large number of heterocyclic compounds (Fig. 2). The equation of the regression line with halogen, sulfur, carboxyl and cyano purines included is:

$$\log V_e^0 = -0.544(\pm 0.183) + 0.344(\pm 0.056)E \qquad (r = 0.750;\ n = 31).$$

The correlation between log V_e^0 of purines only and their delocalization energies shown in Fig. 3 gave the following regression equation:

$$\log V_e^0 = -2.00(\pm 0.46) + 0.707(\pm 0.128)E \qquad (r = 0.879;\ n = 11).$$

Restriction of the correlation to purines with the lactim structure gave a very good correlation:

$$\log V_e^0 = -3.12(\pm 0.40) + 0.987(\pm 0.103)E \qquad (r = 0.984;\ n = 5).$$

Inclusion of purines with a single lactam structure had little effect on this correlation. These results indicate that aromatic character is important for adsorption of purines to Sephadex.

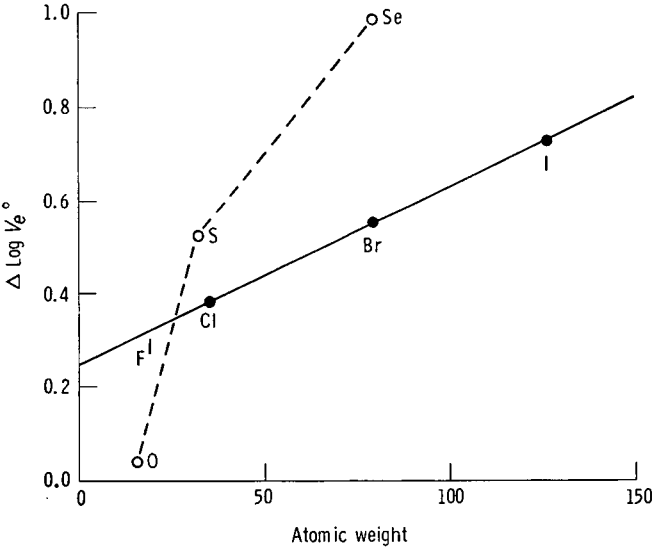

Fig. 1. Relationship between the atomic weights of the halogens (solid line) and chalcogens (dashed line) and their $\Delta \log V_e^0$ values for the 6 position of the purine ring. F^1 is a predicted value.

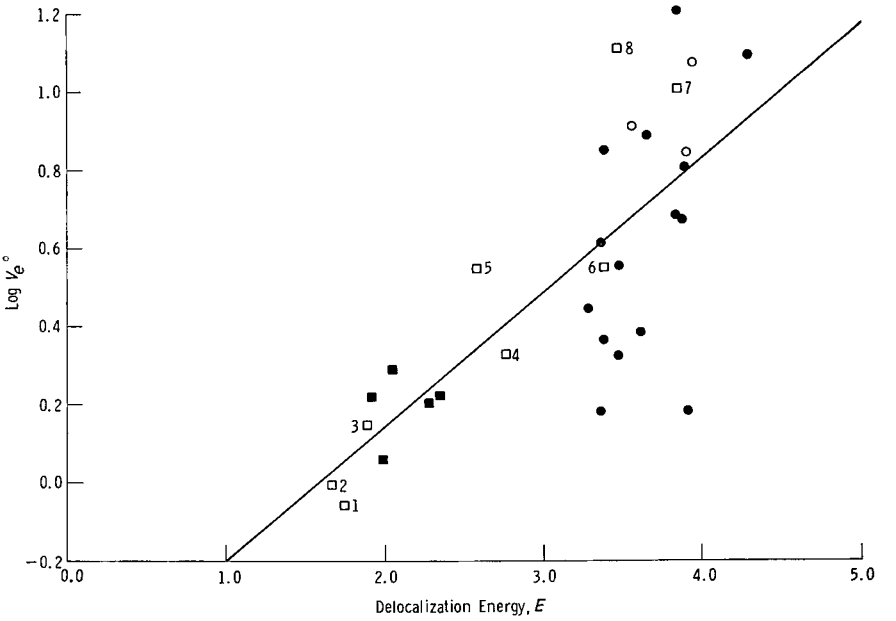

Fig. 2. Correlation between log V_e^0 and delocalization energy, E, for heterocyclic compounds. ●, purines; ○, 8-azapurines; ■, pyrimidines; □ other. 1 = creatinine; 2 = imidazole; 3 = allantoin; 4 = urocanic acid; 5 = 4-amino-5-imidazolecarboxamide; 6 = tryptophan; 7 = 4-aminopyrazolo[3,4-*d*]pyrimidine; 8 = benzimidazole.

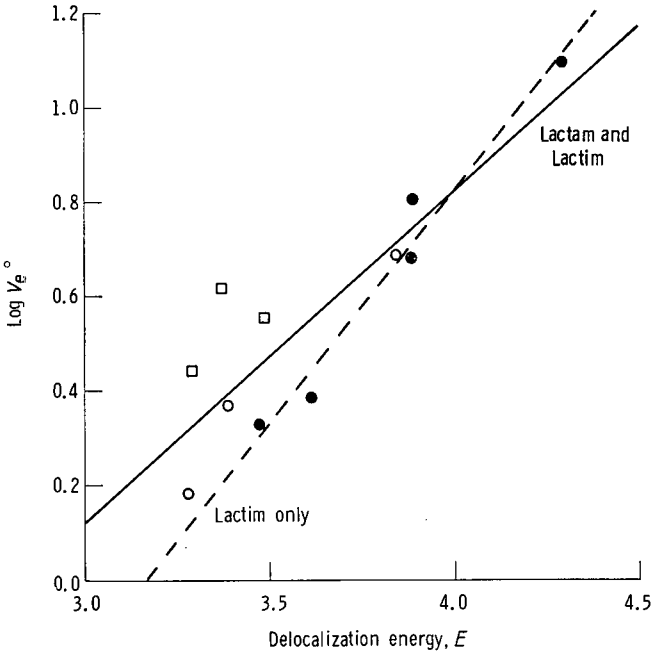

Fig. 3. Correlation between log V_e^0 and delocalization energy, E, for purines. Solid line: ●, lactim; ○, monolactam; □, di- and tri-lactam. Dashed line: ●, lactim only.

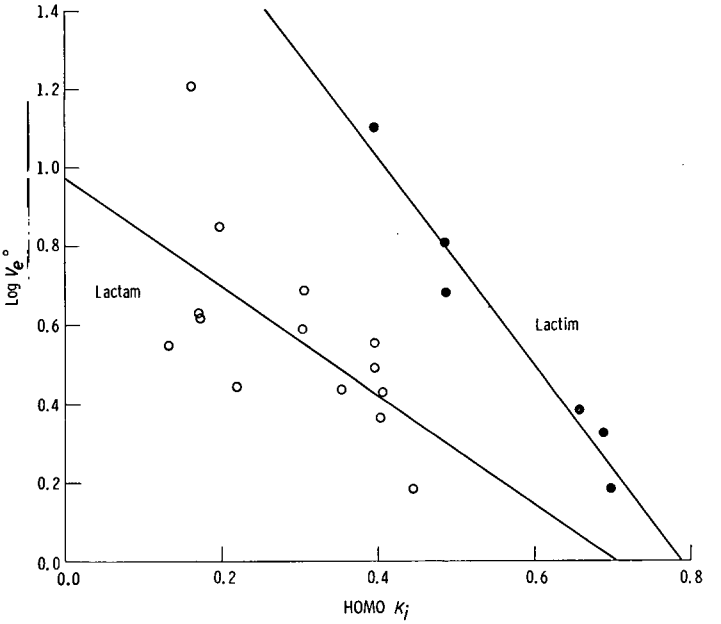

Fig. 4. Correlation between log V_e^0 and HOMO K_i for purines. π-electron donating ability decreases as HOMO K_i increases. ●, lactim; ○, lactam.

Another electronic property obtained from MO calculations is the energy coefficient of the highest occupied molecular orbital, HOMO K_i, which is proportional to the ionization potential of the molecule and inversely proportional to the π-electron donating ability. As shown in Fig. 4, an excellent negative correlation between log V_e^0 and HOMO K_i for purines with the lactim structure was found:

$$\log V_e^0 = 2.08(\pm 0.16) - 2.64(\pm 0.28)\text{HOMO } K_i \qquad (r = 0.977;\ n = 6).$$

However, for purines with one or more lactam structures, the correlation was poorer:

$$\log V_e^0 = 0.978(\pm 0.149) - 1.39(\pm 0.48)\text{HOMO } K_i \qquad (r = 0.642;\ n = 14).$$

In both cases, the slopes were negative, indicating that adsorption to Sephadex increases with increasing π-electron donating ability of the purines. In addition to donating electrons, purines are capable of accepting electrons. The energy coefficient of the lowest empty molecular orbital, LEMO K_i, is inversely proportional to the electron affinity of the molecule, *i.e.*, the electron accepting ability decreases as LEMO K_i becomes more negative. As shown in Fig. 5, increasing adsorption to Sephadex correlated with decreasing electron accepting ability. Again the purines fell into the classes, *viz.* lactim:

$$\log V_e^0 = -2.13(\pm 0.40) - 3.46(\pm 0.51)\text{LEMO } K_i \qquad (r = 0.959;\ n = 6),$$

and lactam:

$$\log V_e^0 = -0.355(\pm 0.240) - 0.837(\pm 0.239)\text{LEMO } K_i \qquad (r = 0.869;\ n = 6).$$

Purines with a lactim structure gave a higher correlation coefficient and a larger slope in the LEMO K_i and adsorption correlation than lactam purines. Thus, the

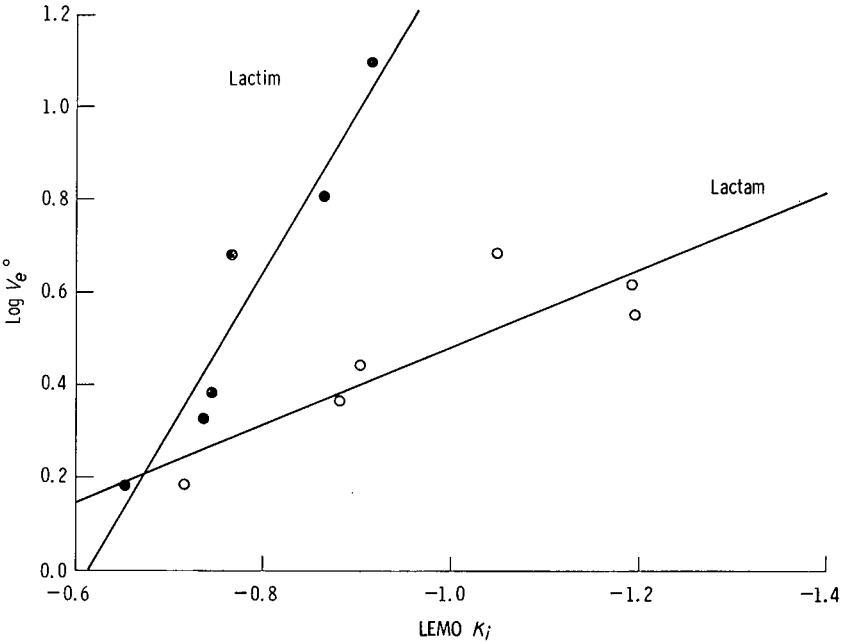

Fig. 5. Correlation between log V_e^0 and LEMO K_i for purines. π-electron accepting ability decreases as LEMO K_i becomes more negative. ●, lactim; ○, lactam.

correlations between the electronic properties of purines and their adsorption to Sephadex indicate that high aromatic character, high electron donating ability, and low electron accepting ability favor adsorption, particularly for purines with the lactim structure.

In addition to these general properties of the π-electrons of the purine rings, the charge density at specific positions of the ring may be of importance, as for example, the unshared electron pairs of the ring nitrogens. The large negative $\Delta \log V_e^0$ values for substitution of a methyl group on the ring nitrogens, especially at the 3,7, and 9 positions, indicate the importance of these nitrogens for adsorption. The basic pK_a values of the purines are proportional to the ease of protonization of the nitrogens and hence proportional to the electron density of the nitrogens. Basic and acidic pK_a values for the purines were obtained from the literature[22-24]. As shown in Fig. 6B, there is an excellent correlation between basic pK_a values and log V_e^0

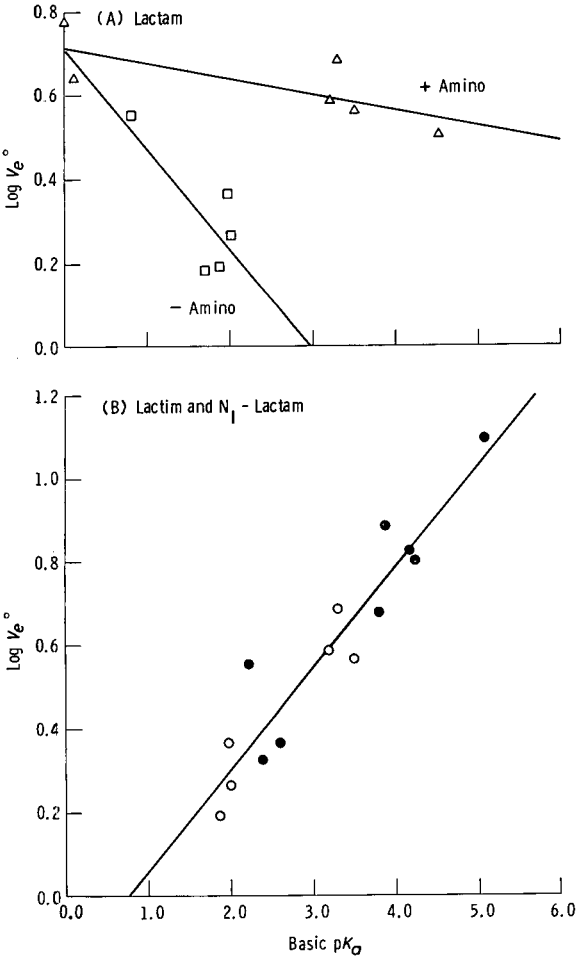

Fig. 6. Correlation between log V_e^0 and basic pK_a for purines. (A) □, lactam without an amino group; △, lactam with an amino group. (B) ●, lactim; ○, N_1-lactam.

values for purines with the lactim structure and those with a single lactam structure involving the nitrogen at the 1 position:

$$\log V_e^0 = -0.189(\pm 0.084) + 0.246(\pm 0.025) \text{ basic } pK_a \quad (r = 0.941;\ n = 14).$$

The inclusion of purines with a lactam structure at the 1 position with the lactim purines is justified by the $\Delta \log V_e^0$ data for methyl substitution, which indicates that the 1 position contributes little to adsorption. However, the purines with one or more lactam structures show a negative correlation between $\log V_e^0$ and basic pK_a (Fig. 6A). For purines without an amino group:

$$\log V_e^0 = 0.705(\pm 0.198) - 0.235(\pm 0.115) \text{ basic } pK_a \quad (r = 0.764;\ n = 5),$$

and for purines with an amino group:

$$\log V_e^0 = 0.718(\pm 0.049) - 0.0378(\pm 0.0164) \text{ basic } pK_a \quad (r = 0.755;\ n = 6).$$

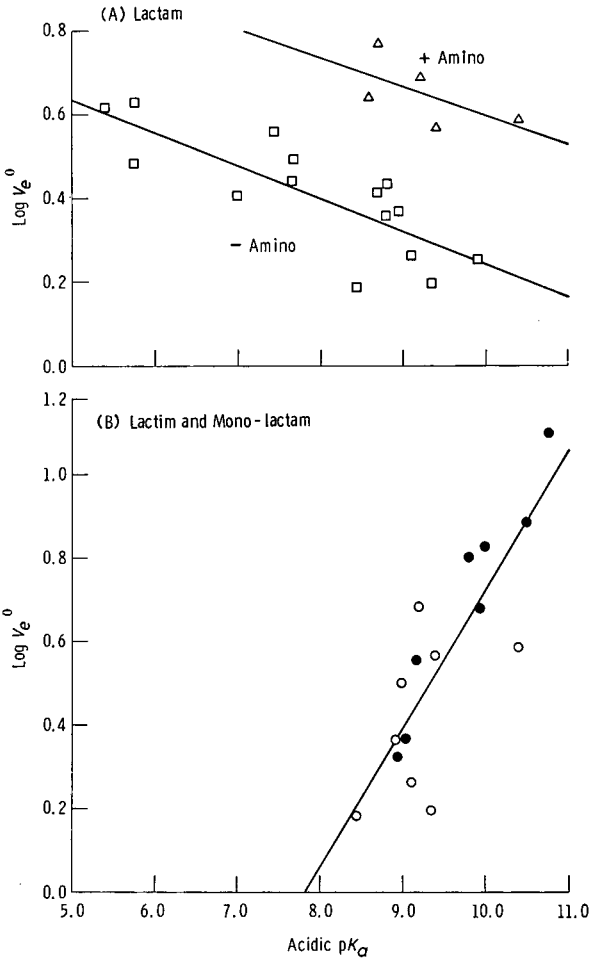

Fig. 7. Correlation between $\log V_e^0$ and acidic pK_a for purines. (A) □, lactam without an amino group; △, lactam with an amino group. (B) ●, lactim; ○, N_1- or N_3-lactam.

The log V_e^0 values were determined at pH 7.0, above the basic pK_a values of the purines, so that actual protonation of the purines is not a consideration in these correlations.

Another measure of the electron density of the ring nitrogens is the anionic pK_a, which is inversely proportional to the ease of removal of a proton from nitrogens with a hydrogen group, and proportional to the electron density of the nitrogens. Fig. 7B shows a good correlation between anionic pK_a and log V_e^0 for purines with the lactim structure and those with a single lactam structure:

$$\log V_e^0 = -2.61(\pm 0.56) + 0.333(\pm 0.059) \text{ anionic p}K_a \qquad (r = 0.834; n = 16).$$

The correlation with purines containing no lactam structure is even higher with $r = 0.959$ ($n = 8$). For purines with one or more lactam structures, there is a negative correlation between anionic pK_a and log V_e^0 (Fig. 7A). For purines without an amino group:

$$\log V_e^0 = 1.03(\pm 0.13) - 0.0794(\pm 0.0164) \text{ anionic p}K_a \qquad (r = 0.802; n = 15),$$

and for purines with an amino group:

$$\log V_e^0 = 1.30(\pm 0.48) - 0.0699(\pm 0.0516) \text{ anionic p}K_a \qquad (r = 0.616; n = 5).$$

Thus, increased electron density on the ring nitrogens, whether measured by increasing basic or acidic pK_a, correlates with increased adsorption to Sephadex for purines with the lactim structure and those with a single lactam structure, but correlates with decreased adsorption for purines with lactam structures. High electron density of purines with the lactim structure may contribute to adsorption by increasing the hydrogen bonding of the nitrogens with unshared electron pairs to the hydroxyl groups of Sephadex. However, for purines with lactam structures, high electron density could decrease adsorption by reducing the hydrogen bonding of lactam hydrogen groups to the oxygen groups of Sephadex.

The preceding correlations of the electronic properties of the purines and their log V_e^0 values reflect relationships between these electronic properties and the difference in standard chemical potentials of the adsorbed molecules and those in solution, *i.e.*, $\mu^0_{liq} - \mu^0_{gel}$. Assuming that μ^0_{liq} is comparable for most purines, adsorption would be related mainly to changes in μ^0_{gel}. However, this assumption may not be valid, and differences in chemical potential could be due to differences in μ^0_{liq} rather than μ^0_{gel}. The standard chemical potentials of purines in solution can be related to their water solubilities. The logarithm of the solubility is inversely proportional to the standard chemical potential difference between the solid and the solution. Assuming that the μ^0 of solid purines are similar, the logarithms of the solubilities are inversely proportional to μ^0_{liq}. Further assuming that μ^0_{gel} is comparable for purines, a negative correlation between log V_e^0 and log solubilities would be expected. For these correlations, the solubilities reported by ALBERT AND BROWN[21] were expressed as μg purine per g water (20°) and were not corrected for molecular weight differences. A good negative correlation between log solubility and log V_e^0 can be seen in Fig. 8, with the regression equation for lactim purines:

$$\log V_e^0 = 1.68(\pm 0.22) - 0.204(\pm 0.046) \text{ log solubility} \qquad (r = 0.840; n = 10),$$

and that for lactam purines:

$$\log V_e^0 = 0.941(\pm 0.114) - 0.156(\pm 0.036) \text{ log solubility} \qquad (r = 0.885; n = 7).$$

When the pyrimidines with lactam structures are included with the lactam purines the correlation coefficient becomes 0.913. The difference between the lines for lactim and lactam purines presumably reflects the lower μ^0_{gel} of the lactim structures with their greater aromatic character. The correlations with solubility suggest that the extent of adsorption of purines to Sephadex is greatly affected by competition between water molecules and the gel surface for the bonding sites of the purines.

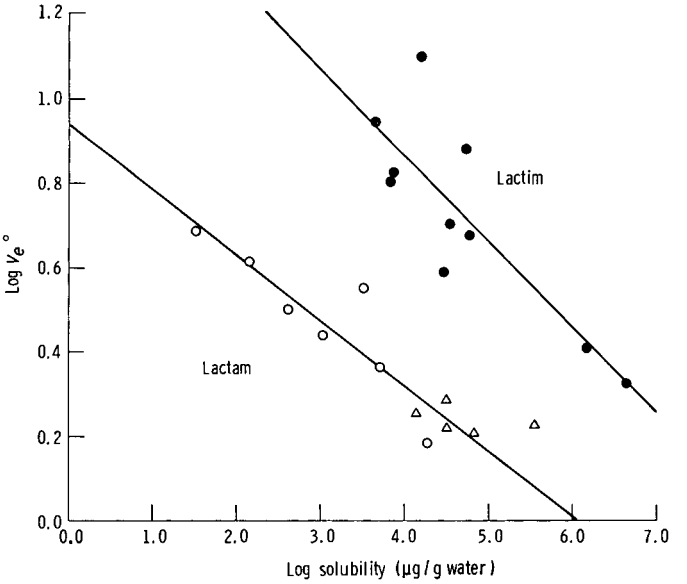

Fig. 8. Correlation between log V_e^0 and the logarithm of the solubility (μg/g water) of purines and pyrimidines. ●, lactim purines; ○, lactam purines; △, lactam pyrimidines.

Turning to the properties of Sephadex which contribute to adsorption of purines the correlations of electron density and adsorption of purines suggest that lactim purines may hydrogen bond to the hydroxyl groups and lactam purines may hydrogen bond to the ether and hydroxyl oxygens of Sephadex. To assess the importance of these groups of the Sephadex G-10 gel for adsorption, purines and heterocyclic compounds were chromatographed on Sephadex LH-20. This is Sephadex G-25 that has been reacted with propylene oxide so that about 60% of the hydroxyl groups have become hydroxypropyl ether groups[25]. Thus, the number of hydroxyl groups is unchanged but the number of ether groups has been greatly increased.

V_e^0 values cannot be used for comparing adsorption to Sephadex G-10 and LH-20 because the two gels have different densities and hence different adsorption capacities per unit column volume and also different internal volumes. Therefore, a new parameter, K_{ad}, was defined which expresses adsorption per unit volume of gel and is independent of column dimensions:

$$K_{ad} = \frac{V_e - (V_0 + V_i)}{V_g} = \frac{V_e - V_e^A}{V_t - V_e^A}$$

where V_g equals gel volume, V_t equals total column volume calculated from the dimensions of the column, and the elution volume of acetone, V_e^A, was used as

($V_0 + V_i$). K_{ad} equals zero for a nonadsorbed compound and increases with increasing adsorption. Another function used to compare the two gels was the affinity number, AN, of DETERMANN AND WALTER[18], which expresses adsorption per weight fraction of the dry gel in the gel bed (w):

$$AN = \frac{(V_e/V_e^G) - 1}{w}$$

where V_e^G is the elution volume of glucose. For the calculation of AN, the elution volume of acetone was used in place of that for glucose.

Table VI gives the column dimensions for Sephadex G-10 and LH-20 and the elution parameters for some purines and other heterocyclic compounds. If adsorption of lactim purines and pyridine is due to hydrogen bonding with the hydrogen of the hydroxyl groups of Sephadex G-10, there should be identical adsorption of these compounds to Sephadex G-10 and LH-20 since both gels have the same proportion of hydroxyl groups. Both K_{ad} and AN values indicate that there is no significant difference between adsorption of pyridine and lactim purines to the two gels (Table VI). If adsorption of lactam purines and pyrrole is primarily due to hydrogen bonding with ether oxygen groups, there should be increased adsorption of these compounds to Sephadex LH-20. But, as shown in Table VI, neither xanthine nor

TABLE VI

COMPARISON OF ADSORPTION OF PURINES AND OTHER HETEROCYCLIC COMPOUNDS TO SEPHADEX G-10 AND LH-20

Volume parameter	*Column volumes*			
	G-10		*LH-20*	
	ml	% *of column volume*	*ml*	% *of column volume*
V_t	78.5	100.0	39.3	100.0
$V_0 + V_i$ (V_e acetone)	61.0	77.7	35.4	90.1
V_0 (V_e dextran)	32.5	41.4	14.3	36.4
V_i	28.5	36.3	21.1	53.7
V_g	17.6	22.4	3.9	9.9
w	0.50		0.25	

Compound	V_e *(ml)*		K_{ad}		AN	
	G-10	*LH-20*	*G-10*	*LH-20*	*G-10*	*LH-20*
Lactim purines						
Purine	106	50.0	2.56	3.74	1.48	1.65
Adenine	251	88.4	10.80	13.59	6.23	5.99
Lactam purines						
Hypoxanthine	113	47.9	2.95	3.20	1.70	1.41
Guanine	199	64.8	7.84	7.54	4.52	3.32
Xanthine	156	55.5	5.40	5.15	3.12	2.27
Heterocyclic						
Pyridine	105	50.4	2.48	3.85	1.43	1.70
Pyrimidine	70	38.3	0.52	0.74	0.30	0.33
Pyrrole	173	59.4	6.38	6.15	3.68	2.71
Pyrazole	117	50.7	3.14	3.92	1.84	1.73
Imidazole	73	34.9	0.68	0.00	0.39	−0.06

pyrrole is more strongly adsorbed to Sephadex LH-20. These results suggest that, unlike substituted benzene derivatives, purines and heterocyclic compounds do not interact with the ether groups of Sephadex gels.

DETERMANN AND WALTER[18] have shown by equilibrium dialysis that phenol is adsorbed to polyethylene glycol but not to dextran and, therefore, that the adsorption of phenol to Sephadex involves the ether cross-links and not the dextran polymer. To study further the importance of ether cross-links of Sephadex for adsorption of purines, equilibrium dialysis binding of adenine and xanthine to

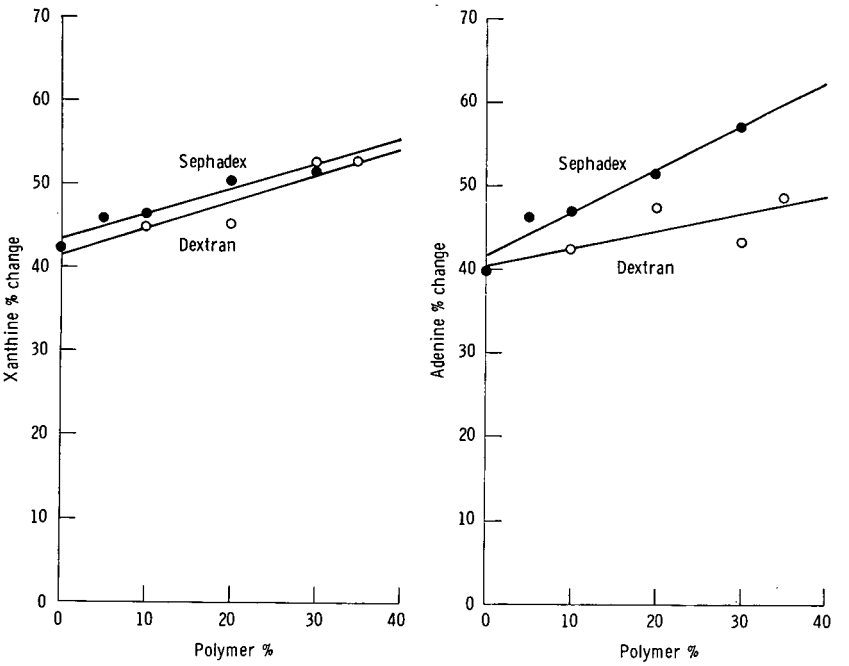

Fig. 9. Per cent change in concentration of xanthine (lactam) and adenine (lactim) after equilibrium dialysis against various concentrations (% w/w) of Dextran T-40 solutions (○) or Sephadex G-10 suspensions (●).

Dextran T-40 and Sephadex G-10 was determined as described in METHODS. Fig. 9 shows the per cent change in concentration of the purines in the dialysis solution as a function of the per cent of polymer in the dialysis tubing. The change in concentration of about 40% for dialysis against buffer is greater than the change of 33% expected from simple dilution, suggesting that the purines were slightly adsorbed to the dialysis tubing. This is consistent with the report of adsorption of uric acid to cellophane[26]. Xanthine was adsorbed equally to dextran and Sephadex, the slopes of the regression lines in Fig. 9 being 0.317 ± 0.066 (S.E.) and 0.297 ± 0.042, respectively. Adenine was adsorbed to dextran to a similar extent with a slope of 0.212 ± 0.091 but was much more strongly adsorbed to Sephadex with a slope of 0.521 ± 0.066. The ratio of the slopes of xanthine over adenine for dialysis against Sephadex G-10 was 0.57. This agreed well with the ratio of V_e^0 for xanthine to adenine of 0.56 from column

chromatography on Sephadex G-10, indicating that comparisons between dialysis and chromatographic results are valid.

These equilibrium dialysis results indicate that, unlike phenol, purines are adsorbed to the dextran portion of Sephadex. The equal adsorption of xanthine to dextran and Sephadex indicates that hydrogen bonding of lactam hydrogens to ether groups does not contribute to the adsorption to Sephadex. However, the stronger adsorption of adenine to Sephadex compared to dextrans suggests that the amino group may form hydrogen bonds with the ether cross-links of Sephadex as does the phenol group.

DISCUSSION

The following properties of purines correlated with increasing adsorption to Sephadex G-10: increasing π-electron delocalization energy, increasing π-electron donating ability, decreasing π-electron accepting ability, decreasing water solubility, and increasing electron density at the ring nitrogens as measured by basic and acidic pK_a for purines with lactim structures, but decreasing electron density at ring nitrogens for purines with lactam structures. These correlations suggest that the mechanism of adsorption of purines to Sephadex involves several types of molecular interactions such as weak hydrogen bonding between π-electrons and hydroxyl groups of Sephadex, hydrogen bonding of nitrogens with unshared electron pairs to hydroxyl groups of Sephadex, hydrogen bonding of purine lactam hydrogens to hydroxyl oxygens of Sephadex and hydrogen bonding of amino groups to ether oxygens of Sephadex. In addition, because of the polarizability of the π-electrons and the dipole moments of purines[27], dipole–dipole, dipole-induced dipole and dispersion forces may also play a role in the adsorption of purines to Sephadex. Furthermore, the correlations of elution volumes with decreasing water solubility indicate that competition between water molecules and the Sephadex gel for bonding sites of the purines plays an important role in chromatography of purines on Sephadex.

ACKNOWLEDGEMENTS

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REFERENCES

1 P. FLODIN, *Dextran Gels and their Applications in Gel Filtration*, Dissertation, Uppsala, Sweden, 1962.
2 B. GELOTTE, *J. Chromatogr.*, 3 (1960) 330.
3 S. ZADRAŽIL, A. ŠORMOVÁ AND F. ŠORM, *Coll. Czech. Chem. Commun.*, 26 (1961) 2643.
4 TH. HOHN AND W. POLLMAN, *Z. Naturforsch.*, 18b (1963) 919.
5 R. BRAUN, *Biochim. Biophys. Acta*, 142 (1967) 267.
6 R. BRAUN, *Biochim. Biophys. Acta*, 149 (1967) 601.
7 L. SWEETMAN AND W. L. NYHAN, *J. Chromatogr.*, 32 (1968) 662.
8 J. LERNER AND A. I. SCHEPARTZ, *J. Chromatogr.*, 39 (1969) 132.
9 C. WASTERNACK AND H. REINBOTHE, *J. Chromatogr.*, 48 (1970) 551.

10 J. DE BERSAQUES, *J. Chromatogr.*, 31 (1967) 222.
11 G. GORBACH AND J. HENCKE, *J. Chromatogr.*, 37 (1968) 225.
12 J. X. KHYM AND M. UZIEL, *J. Chromatogr.*, 47 (1970) 9.
13 A. J. W. BROOK, *J. Chromatogr.*, 47 (1970) 100.
14 J. B. WOOF AND J. S. PIERCE, *J. Chromatogr.*, 28 (1967) 94.
15 J. A. DEMETRIOU, F. M. MACIAS R., M. J. MCARTHUR AND J. M. BEATTIE, *J. Chromatogr.*, 34 (1968) 342.
16 A. J. W. BROOK AND S. HOUSLEY, *J. Chromatogr.*, 41 (1969) 200.
17 A. J. W. BROOK AND K. C. MUNDAY, *J. Chromatogr.*, 47 (1970) 1.
18 H. DETERMANN AND I. WALTER, *Nature*, 219 (1968) 604.
19 A. PULLMAN AND B. PULLMAN, *Quantum Biochemistry*, Wiley, New York, 1963.
20 B. PULLMAN, *Biopolymers Symposia*, No. 1 (1964) 141.
21 A. ALBERT AND D. J. BROWN, *J. Chem. Soc.*, (1954) 2060.
22 S. F. MASON, *J. Chem. Soc.*, (1954) 2071.
23 E. H. RODD, *Chemistry of Carbon Compounds*, Vol. IVc, Elsevier, Amsterdam, 1956.
24 B. WEISSMAN, P. A. BROMBERG AND A. B. GUTMAN, *J. Biol. Chem.*, 244 (1957) 407.
25 B. BUSH AND T. E. L. JONES, *J. Chromatogr.*, 49 (1970) 448.
26 P. A. SIMKIN, *J. Chromatogr.*, 47 (1970) 103.
27 B. PULLMAN, P. CLAVERIE AND J. CAILLET, *Science*, 147 (1965) 1305.

CHROM. 5354

SEPARATION OF 1-DIMETHYLAMINO-NAPHTHALENE-5-SULPHONYLAMIDES BY GEL CHROMATOGRAPHY

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SUMMARY

For the determination of biogenic amines as their 1-dimethylamino-naphthalene-5-sulphonyl derivatives in tissue samples it is often necessary to remove 1-dimethylamino-naphthalene-5-sulphonylamides and the 1-dimethylamino-naphthalene-5-sulphonyl derivatives of some other amines present in large amounts in the tissue. Moreover a pre-separation of the complex mixture of amines is desirable. We recommend the pre-separation of dansylated tissue extracts by chromatography on molecular sieves (Sephadex LH-20). The method is universal in its applicability, simple and has a number of advantages over other pre-separation methods.

INTRODUCTION

The identification of biogenic amines in the form of their 1-dimethylamino-naphthalene-5-sulphonyl (DANS) derivatives is normally performed after their separation by thin-layer chromatography (TLC)[1–4]. The concentrations of biogenic amines in tissues are generally very low, and the amounts vary over a wide range. Ammonia and the polyamines spermidine and spermine, are often present in much greater amounts than the other amines, so that they cause trouble in the TLC, particularly, when large amounts of tissue have to be worked up for the determination of certain amines. The high concentration of γ-aminobutyric acid (GABA) can also give overlapping spots, if dansylated brain or retina extracts are separated on thin-layer chromatograms. The DANS derivative of GABA moves together with the DANS-amides[5].

Dansylation also gives rise to side reactions. One of the chief side reaction products is DANS-dimethylamide[4], which is produced in a fairly high concentration, if excessive amounts of DANS-Cl are used for completion of the dansylation reaction. Owing to this it is necessary, in many cases, to apply pre-separation methods before certain amines can be determined in tissues, in spite of the sensitivity of the dansyl method and the effectiveness of TLC on Silica Gel G for the separation of DANS derivatives.

In principle the whole spectrum of chromatographic and electrophoretic techniques is applicable for the pre-separation of the tissue extracts before dansylation.

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However, in some cases certain dansyl derivatives can be concentrated from a dansylated tissue extract on account of their specific properties; *e.g.* in the determination of GABA and related compounds[6,7].

On the basis of our experience with the dansylation method it seemed desirable to develop a generally applicable pre-separation method for dansylated tissue extracts. Gel column chromatography recommended itself for this purpose, since separations roughly according to molecular weight could be expected. The molecular weights of the DANS-derivatives of biogenic amines are in the range of 250 (DANS-NH_2) to 1134 (tetra-DANS-spermine).

In the present paper, the chromatographic behaviour of some DANS derivatives on Sephadex LH-20 gel columns is described and the applicability of gel column chromatography to the determination of biogenic amines from tissues, in form of their DANS derivatives, is discussed.

MATERIALS AND METHODS

DANS-amides were prepared in our laboratory on a preparative scale. They moved on thin-layer chromatograms as single spots in many different solvent systems and were further characterized by elemental analysis[3,8] and mass spectrometry[9].

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Solvents were obtained from E. Merck, Darmstadt, G.F.R., and were of analytical grade.

Sephadex LH-20 was allowed to swell in an excess of solvent for at least 3 h with occasional stirring with a glass rod. The suspension of the gel in solvents with a low specific gravity were poured into ordinary chromatographic glass columns (0.9 × 120 cm) in the usual manner (see the instructions by Pharmacia for the preparation of gel columns). The height of the gel bed was: 100 cm; the flow rate was 10 ml/h. Gel suspensions in chloroform were poured into glass columns (2 × 100 cm) with special PTFE end assemblies with porous polyethylene support discs, which enabled us to use the column for ascending or descending chromatography. Height of the gel bed was: 70 cm; flow rate, 30 ml/h.

Both 1.5- and 2.5-ml fractions of the eluates were collected in test tubes by means of an automatic fraction collector (FRS 63/E, Serva, Heidelberg, G.F.R.).

For the identification of the DANS derivatives, aliquots of the fractions were applied to Silica Gel G plates (layer thickness 200 μ). The chromatograms were developed by ascending chromatography in normal solvent vapour saturated tanks. The solvents were: trichloroethylene–methanol (95:5); diethyl ether–cyclohexane (90:10); benzene–cyclohexane–methanol (85:15:2)[3]. The fluorescent spots were observed under a UV lamp (365 nm).

RESULTS

Preliminary experiments demonstrated that the separation of DANS-NH_2 from most of the DANS derivatives of the natural amines is possible on gel columns filled with Sephadex G-10, if alcohol–water mixtures were used for swelling the gel. However, the separation of the different DANS-amines from each other was rather poor in this system. Better separations were obtained with LH-20 in ethyl acetate, methanol

and acetone. But really satisfactory separations were only observed when dioxane was used as solvent. The only important disadvantage of using dioxane is the formation of peroxides, which can destroy the DANS-amides. Table I shows the elution volumes obtained on elution of DANS-amides from a Sephadex LH-20 column with dioxane.

TABLE I

MOLECULAR WEIGHTS OF DANS-AMIDES AND -PHENOL ESTERS AND THEIR ELUTION VOLUMES ON SEPHADEX LH-20 COLUMNS

Column A: 0.9 × 120 cm; gel bed volume, 63 ml; flow rate, 10 ml/h; solvent, dioxane. Column B: 2 × 100 cm; gel bed volume, 220 ml; flow rate, 30 ml/h; solvent, chloroform.

Compound	*Mol. wt.*	*Elution volumes (ml)*	
		Column A	*Column B*
Tetra-DANS-spermine	1134	47	90
Tri-DANS-adrenaline	882	51	106
Tri-DANS-spermidine	845	51	106
Bis-DANS-5-hydroxy-tryptamine (serotonin)	642		200
Bis-DANS-pyridoxamine	634	66	118
Bis-DANS-cysteamine	618	56	
Bis-DANS-tyramine	603	59	114
Bis-DANS-histamine	577	58	114
Bis-DANS-putrescine	554	59	145
Bis-DANS-N-methyl-trimethylenediamine	554		110
Bis-DANS-trimethylenediamine	540	59	
Mono-DANS-glucosamine	412	100	>1000
Mono-DANS-hordenine	398	62	119
Mono-DANS-tryptamine	393	69	223
Mono-DANS-β-hydroxy-β-phenylethylamine	370	69	
Mono-DANS-adenine	368	56	280
Mono-DANS-β-phenylethylamine	354	64	132
Mono-DANS-phenol	327	61	
Mono-DANS-piperidine	318	62	
Mono-DANS-pyrrolidone[a]	318	70	121
Mono-DANS-imidazole	301	69	
Mono-DANS-ethanolamine	294	77	400
Mono-DANS-dimethylamine	278	66	121
Mono-DANS-methylamine	264	72	
DANS-NH_2	250	94	600

[a] Reaction product of GABA with excess DANS-Cl.

On account of drawbacks due to peroxide formation in dioxane we looked for other useful solvents. Chloroform was found to give the best separations. Since, however, chloroform has a higher specific gravity than the gel in the swollen state, it was necessary to use chromatographic columns with adjustable end assemblies. Table I shows the elution volumes obtained for some DANS-amides in LH-20–chloroform.

DISCUSSION

The elution volumes of the DANS derivatives obtained by Sephadex LH-20 gel chromatography do not, however, correspond to their molecular weights. There are significant interactions between the DANS derivatives and the gel matrix, which influence the elution volumes considerably. These interactions are obviously de-

pendent on the polarity of the solvent. In methanol for instance, DANS-dimethylamide is eluted from the column before even tetra-DANS-spermine and tri-DANS-spermidine, because the latter molecules are reversibly adsorbed on the matrix.

A decrease in the dielectric constant of the solvent generally improves the separation of the DANS derivatives on LH-20, provided that the gel swells sufficiently. Dielectric constants and gel volumes per g dry gel after swelling in the solvents, we have studied, are summarized in Table II. The ratios between gel volume and dielectric constant are maximal in the case of dioxane and chloroform, *i.e.* for those solvents which effected the best separations. It seems that the magnitude of the gel volume/dielectric constant ratio is to some extent a measure for the efficacy of the separating system for DANS derivatives.

TABLE II

DIELECTRIC CONSTANTS[10] OF THE SOLVENTS AND GEL VOLUME PER G DRY GEL OF SEPHADEX LH-20 IN DIFFERENT SOLVENTS

Solvent	*Dielectric constant, D*	*Gel volume per g dry gel (ml/g)*	*Ratio of gel volume per g dry gel/dielectric constant (ml/g·D)*
Dioxane	3.0	3.6	1.20
Diisopropyl ether	3.9	1.5	0.38
Chloroform	5.1	4.2	0.82
Ethyl acetate	6.1	2.4	0.39
Butanone-2	10.0	2.5	0.14
Cyclohexanone	18.2	2.9	0.16
Acetone	21.5	2.9	0.13
Propanol-2	26.0	3.8	0.15
Methanol	31.2	4.5	0.14

With non-polar solvents DANS-NH_2, DANS-ethanolamine and other hydroxyl-group containing compounds are strongly retained by the gel column (see Table I). This effect of non-polar solvents together with the rapid elution of tetra-DANS-spermine and tri-DANS-spermidine is of great importance in the application of the gel column to the separation of dansylated tissue extracts, since the main sources of interference with the TLC can thus be eliminated very simply. It is possible to remove DANS-NH_2 nearly completely by chromatography of a dansylated extract of 1–2 g tissue even with a gel column 1 × 15 cm. However, the separation of DANS-dimethylamide from some of the other significant components is not possible by gel chromatography, as can be seen from the values in Table I. This compound overlaps, among other compounds, the DANS derivative of GABA, piperidine, hordenine and partially overlaps β-phenylethylamine as well.

The use of our pre-separation method for DANS derivatives has a number of advantages in comparison to other pre-separation methods, provided that no specific method for the concentration of a particular amine is available. They are:

(1) Large amounts of tissue can be worked up without difficulty, since the capacity of the gel column is high. This is especially of interest for the identification of amines by mass spectrometry, since larger samples are necessary for this method than for the quantitation of the DANS derivatives by fluorometry[8,9].

(2) Dansylation prevents certain compounds from autoxidation, *viz.* catecholamines. However, auto-oxidation of these compounds has to be prevented during the dansylation by suitable precautions.

(3) The quantitative extraction of many amines and phenols in form of their DANS derivatives is in many cases simpler than the isolation of the free amines, since DANS-amides and -phenol esters are soluble in non-polar solvents.

(4) The by-products of the dansylation reactions accumulate only in certain fractions of the gel column chromatogram. Because of this the thin-layer chromatograms prepared from gel column chromatography fractions are generally very clean, and therefore they are especially suited for quantitative evaluation. By combination of gel chromatography with suitable TLC separations, quantitative methods can be worked out for practically all those amines and phenols in a tissue sample, which react in a reproducible way with DANS-Cl.

DANS-amino acids are strongly retained by the gel under the conditions described.

REFERENCES

1 N. SEILER AND M. WIECHMANN, *Experientia*, 21 (1965) 203.
2 N. SEILER AND M. WIECHMANN, *J. Chromatogr.*, 28 (1967) 351.
3 N. SEILER AND M. WIECHMANN, in A. NIEDERWIESER AND G. PATAKI (Editors), *Progress in Thin-Layer Chromatography and Related Methods*, Vol. 1, Ann Arbor – Humphrey Science Publishers, Ann Arbor, 1970, p. 94.
4 N. SEILER, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 18, Wiley, New York, 1970, p. 259.
5 N. SEILER AND M. WIECHMANN, *Z. Physiol. Chem.*, 349 (1968) 588.
6 N. SEILER AND M. WIECHMANN, *Z. Physiol. Chem.*, 350 (1969) 1493.
7 N. SEILER, M. WIECHMANN, H. A. FISCHER AND G. WERNER, *Brain Res.*, 28 (1971) 317.
8 N. SEILER AND M. WIECHMANN, *Z. Anal. Chem.*, 220 (1966) 109.
9 N. SEILER, H. SCHNEIDER AND K.-D. SONNENBERG, *Z. Anal. Chem.*, 252 (1970) 127.
10 J. D'ANS AND E. LAX, *Taschenbuch für Chemiker und Physiker*, Springer, Berlin, 1949.

CHROM. 5395

AUTOMATIC LIQUID CHROMATOGRAPHY OF ORGANIC COMPOUNDS

II. ADSORPTION CHROMATOGRAPHY OF BENZENE DERIVATIVES ON STYRENE-BASED CATION-EXCHANGE RESIN*

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SUMMARY

The adsorption chromatography of benzene derivatives on styrene-based cation-exchange resin has been investigated. Adsorption isotherms of benzene derivatives are discussed and the possibility of separation and quantitative treatment is considered for some cases of non-linear isothermal adsorption.

The distribution coefficient and the height equivalent to a theoretical plate, important for separation and quantitative analysis, have been calculated for thirty-eight derivatives of benzene.

INTRODUCTION

DAVIES AND THOMAS[2] and SAMUELSON[3] reported on the adsorption of carboxylic acids on sulfonic acid-type ion-exchange resin. Salting-out chromatography of aromatic compounds has been reported by SARGENT AND RIEMAN[4] and FUNASAKA *et al.*[5,6] The adsorption of aromatic compounds on the skeleton of ion-exchange resins has been considerably investigated[7–10].

Although some relationships between isotherms and liquid chromatograms have been discussed, there has been little experimental study[11]. The experimental design and column standardization are very difficult, and the experimental conditions used must therefore be carefully controlled. Thus the diameters of resin particles should be controlled, the resin should be packed into the column as homogeneously as possible, the extra column volume and the sample injection volume should be decreased, and the column temperature and the flow rate should be maintained constant. Under these controlled conditions, an isothermal curve can be predicted from the variation of the distribution coefficients (K_D) with change of solute concentration.

In this paper, K_D and height equivalent to a theoretical plate (HETP) of thirty-eight derivatives of benzene were obtained, and the variation of retention volumes (V_R) with the change of concentration of several compounds was studied. The relation between the type of adsorption isotherm and the separative effect of admixed solutes in liquid chromatography was demonstrated for phenol derivatives.

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EXPERIMENTAL

Samples

Samples used as solutes were recrystallized or redistilled and their purity was determined by means of the melting point, organic microanalysis and IR spectroscopy. Some of them were kindly supplied by WAKO Pure Chemicals Co., Ltd. The samples were weighed with an accuracy of 10 μg and dissolved in deionized and redistilled water to give solutions of various concentrations. The injection volume of each sample solution was 100 μl.

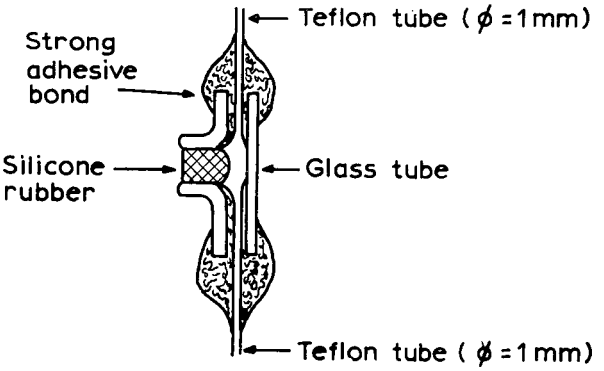

Fig. 1. Special sample injector.

Equipment

A UV-detecting, automatic recording liquid chromatograph was used, fitted with a reverse phase duplex plunger pump, JLC-P_2 (JEOL Co., Ltd.); a constant-temperature water circulator connected to the column jacket, TAIYO Thermo Unit C-550 (TAIYO Scientific Fabrication Co., Ltd.); a specially designed sample injector depicted in Fig. 1; a UV detector, JLC-B_1 UV Detector (JEOL Co., Ltd.), which had four light interference filters (transmittances were about 40 % at 260, 273, 280 and 287 nm) and a quartz flow cell (lightpath 3 mm); and a 6-pen electronic recorder (OHKURA Electric Co., Ltd.). The connector between the column and the flow cell was a 38-cm Teflon tube of 1 mm I.D. that was as short as possible.

The inner diameter of the glass column used was 0.8 cm. The column length was controlled by a variable column plunger. The outlet pressure was controlled by a valve.

Preparation of stationary phase

Dowex 50W X2, 4, 8 and 12, hydrogen form, (each 200–400 mesh), Aminex X12 (21–29 μm), Amberlite CG-120 (X8.5) (400–600 mesh) and Bio-Rad AG 50W X8 (minus 400 mesh) were passed through a sieve to obtain a particle size of minus 400 mesh, and the particle size was further adjusted to be uniform by the sedimentation method described previously[1].

The sodium form of the ion-exchange resin was formed with a large excess of 2 *N* sodium hydroxide solution and washed with water in a long column. The diameters of the particles of both types of resin were tested by microscopy and are tabulated in Table I.

TABLE I

CROSS-LINKING, PARTICLE DIAMETERS AND WEIGHTS IN COLUMNS OF ION-EXCHANGE RESINS USED IN EXPERIMENTS

	Commercially available resin Dowex 50W				*Aminex*	*Amberlite CG-120*	*Bio-Rad AG 50W*
Cross-linking	2	4	8	12	12	(8.5)	8
Mean diameter of particles (μm) (as H^+ type)	80	65	60	60	25	60	28.1
Resin weight (g) (column: 18.5 × 0.8 cm)							
H^+ form	1.544	2.350	3.670	4.387	4.144	3.234	3.735
Na^+ form	1.716	2.816	4.241	4.965	4.747	3.990	4.301

Ion-exchange resin was packed into a column homogeneously by means of a supporting tube which had the same diameter as the column connected to the top of the column, and the supporting tube was then detached. By using such a supporting tube, the column length was proportional to the weight of the packed resin (Fig. 2).

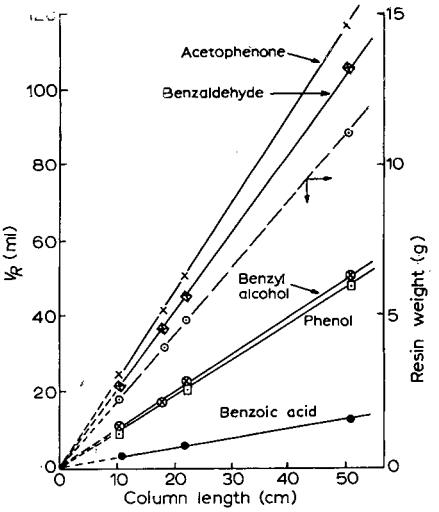

Fig. 2. Correlation of the column length with weight of packed resin and V_R value of some benzene derivatives. Amberlite CG-120, Na^+ form. Mean diameter of particles, 60 μm. Column diameter, 0.8 cm. Eluent: water. Temperature, 40°. Flow rate, 0.45 ml/min.

The variation of V_R values with the change in the degree of cross-linking is shown in Fig. 3A and B. This indicates that the effect of cross-linking on V_R values is an important problem which should be investigated, but in this paper a degree of cross-linking of 8 % was selected.

Measuring conditions

Deionized and redistilled water was run as eluent at rates of 0.45 and 0.36 ml/min. The column temperature was controlled at 30, 40, 50, 52.5, 60 and 70°. The wave-

length of the light interference filter used was selected as near as possible to the absorption maximum of the solute.

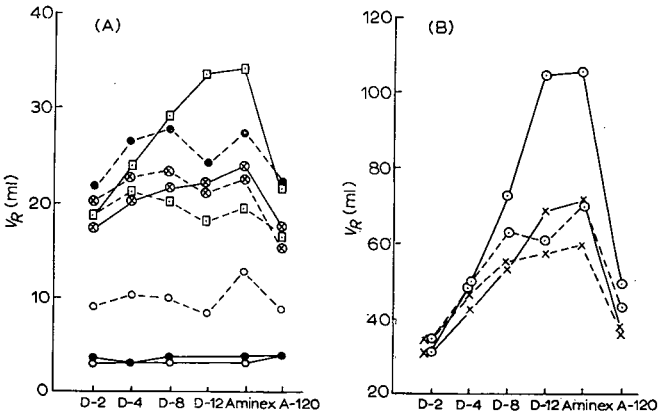

Fig. 3. Variation of V_R values of some benzene derivatives with change of cross-linking, of ion-exchange resins. Column, 18.5 × 0.8 cm. Eluent, water. Flow rate, 0.45 ml/min. Temperature, 52.5°. Symbols of resins: D-2, Dowex 50W X2; D-4, Dowex 50W X4; D-8, Dowex 50W X8; D-12, Dowex 50W X12; Aminex, Aminex X12; A-120, Amberlite CG-120. (———), Na^+ type; (– – – –), H^+ type; ⊡, phenol, ⊗, benzylalcohol; ○, *p*-nitrobenzoic acid, ●, benzoic acid; ×, acetophenone; ⊙, nitrobenzene.

Calibration curve

A linear relation between peak area and solute concentration was obtained. Some solutes gave a non-linear adsorption isotherm, and the calibration curves were almost linear or very slightly curved (Fig. 4A and B).

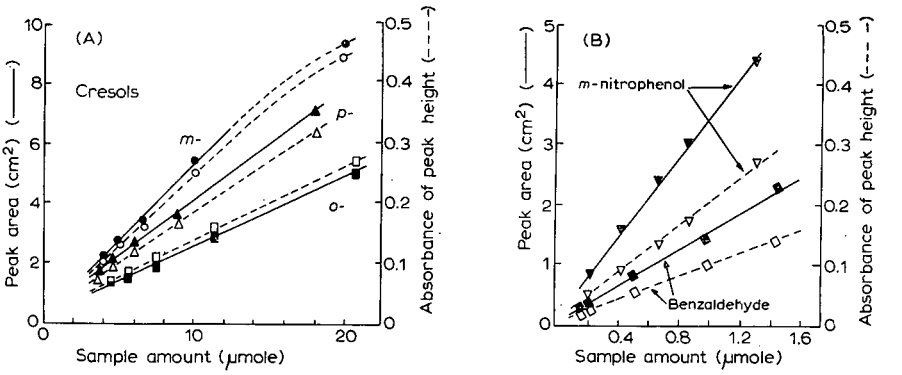

Fig. 4. Calibration curves of benzene derivatives. Bio-Rad AG 50W X8, Na^+ form. (A): *o*-, *m*- and *p*-cresols. (B): *m*-nitrophenol and benzaldehyde.

RESULTS AND DISCUSSION

Usually aqueous solvent systems are used in ion-exchange chromatography. Such systems were used in this work since it is preferable to use an aqueous system

for the investigation of the effect of physical adsorption mechanisms on strong ion-exchange resins.

Linear relations were obtained between the column length and the weight of packed resin and V_R value (Fig. 2). Also, log K_D was proportional to the reciprocal of absolute temperature in the range 30–70°C (as shown in Fig. 5). These facts showed that the adsorption mechanism should be nearly constant under these experimental conditions. V_R values and peak widths for benzene derivatives were determined from their chromatograms at the concentrations given in Tables II and III, and their K_D values and HETP values were calculated from them. Relative standard deviations of V_R values and widths were less than 2.5 % and less than 5 %, respectively ($n \geqslant 5$).

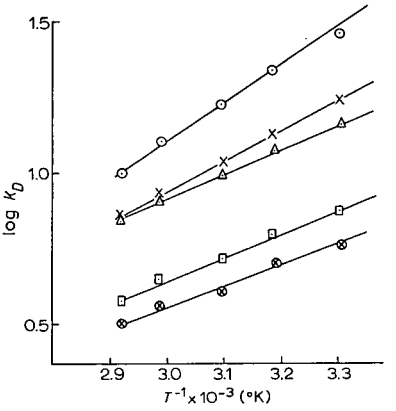

Fig. 5. Plot of log K_D of some benzene derivatives on Bio-Rad AG 50W X8, Na^+ form, against the reciprocal of absolute temperature of the column. ⊗, benzyl acohol; ⊡, phenol; △, *p*-cresol; ×, acetophenone; ⊙, nitrobenzene.

The K_D values on Bio-Rad AG were less than on Dowex and the HETP values on Bio-Rad AG were 20–50 % of those on Dowex. Particle diameters, column temperatures and flow rates are given in Tables II and III.

As benzenesulfonic acid and *p*-toluenesulfonic acid were not adsorbed on the hydrogen form of strong ion-exchange resins, the volume of the mobile phase in the column was made the same as their V_R value. *o*- and *p*-nitrobenzoic acids were sparingly adsorbed and other substituted benzoic acids were adsorbed on the hydrogen form of the resins.

On the other hand, benzenesulfonic acid, *p*-toluenesulfonic acid and benzoic acids[8] were exchanged with sodium ions on the sodium form of strong ion-exchange resins. Accordingly, the sodium salts of these compounds were used as solutes, and the volume of the mobile phase in the column was made the same as the V_R value of sodium benzenesulfonate. Sodium benzoic acids were sparingly adsorbed on the sodium form of the resins.

Nitrophenols were partially exchanged with the sodium form of the resins, as demonstrated by UV spectroscopy. The other solutes in Tables II and III were adsorbed similarly on the hydrogen and sodium forms of the resins.

The simplest mechanism of adsorption is the case in which the adsorption iso-

TABLE II

K_D VALUES AND HETP VALUES OF BENZENE DERIVATIVES ON H^+ FORM BIO-RAD AG 50W AND DOWEX 50W IN LIQUID CHROMATOGRAPHY

UV filters: **, 260 nm; ***, 273 nm; ****, 280 nm. Column: 18.5 × 0.8 cm. A = Bio-Rad AG 50W X8, H^+ form (particle diameter 28 μm). Flow rate, 0.36 ml/min; temperature, 60°. B = Dowex 50W X8, H^+ form (particle diameter 60 μm). Flow rate, 0.45 ml/min; temperature, 52.5°.

$X \cdot C_6H_4 \cdot Y$		Position of Y	UV filter	A			B		
X	Y			Sample amount (μmole)	K_D (ml/g)	HETP (mm)	Sample amount (μmole)	K_D (ml/g)	HETP (mm)
H	SO_3H		**	0.745		0.635			
	COOH		***	2.37	4.97	0.238	2.37	6.38	
	OH		**	2.46	3.63	0.476	44.1	4.35	0.858
	NO_2		**	1.66	11.8	0.156	1.66	14.9	0.522
	CH_2OH		**	35.2	4.51	0.141	35.2	4.92	0.569
	$COCH_3$		****	4.49	9.99	0.314	4.49	12.8	0.679
	CHO		****	2.38	8.56	0.181	3.77		0.734
COOH	NO_2	2	****	0.544	0.680	0.378	4.10	1.17	5.94
		3	****	0.807	3.50	5.65	1.86	4.84	
		4	****	1.25	1.08	2.61	2.51	1.50	6.24
	OH	2	****	0.725	1.01	3.68			
		3	****		2.67	(0.743)			
		4	****	3.62	3.35	(0.455)	3.62	4.26	
	Cl	2	**	12.8	2.77	(2.57)			
		3	**	16.0	5.63	2.29			
	CH_3	3	****		(7.44)	(1.50)			
CH_3	SO_3H	4	**	2.00		0.782	4.00		1.41
	NO_2	2	****	0.216	20.7	0.726			
		3	****	0.363	24.9	0.165	0.363	31.1	0.472
		4	****	0.322	23.1	0.134			
OH	CH_3	2	**	1.70	5.90	0.201	22.7	6.79	1.49
		3	**	1.48	6.09	0.204	20.3	7.46	1.22
		4	**	1.79	6.68	0.145	17.9	7.97	0.987
	iso-C_3H_7	2	***	1.67	11.1	0.210			
		3	***	1.69	12.3	0.182			
		4	***	1.20	13.6	0.220			
	OH	2	****	0.511	2.19	0.235		2.71	
		3	****	0.737	1.95	0.270		2.60	
		4	****	0.602	1.79	0.295	72.8	2.44	
	NO_2	2	**	1.50	9.59	0.0997	1.50	11.8	0.449
		3	**	1.93	8.67	0.156	15.4	11.1	1.01
		4	**	1.71	8.71	0.269	9.49	12.4	0.774
	Cl	2	****	4.88	8.23	0.303	19.6	11.4	0.783
		3	****	5.04	10.1	0.305	17.9	12.9	1.29
		4	****	4.48	10.3	0.202	17.9	13.3	0.866
	Br	2	****	0.647	11.8	0.159			
		3	****	0.601	15.9	0.300			
		4	****	0.688	15.7	0.184			

therm is linear and ideal. Cases of linear ideal isotherms in adsorption phenomena are few and there are many examples of non-linear and non-ideal isotherms.

Chromatograms obtained in this experiment showed leading, tailing or Gaussian curves (some examples are shown in Fig. 6A, B and C). In the case of non-ideal isotherms, it is impossible to obtain the isotherm directly from its chromatogram. In such cases, the variation of the V_R value with change of solute concentration was

measured and its influence examined on separation and quantification. A range of solute concentration in which there was little or no change in the K_D value was found (Fig. 7A and B). In such a concentration range, the design of a separation and quantitative analysis method in non-linear isothermal adsorption chromatography should be practical in some cases. An example of the separation of *o*-, *m*- and *p*-nitrophenol is shown in Fig. 6A. Chromatograms of *o*- and *p*-nitrophenol showed leading, corresponding to an isotherm convex to the horizontal axis, but *m*-nitrophenol showed tailing corresponding to a concave isotherm. The resolution of these chromatograms

TABLE III

K_D VALUES AND HETP VALUES OF BENZENE DERIVATIVES ON THE Na FORM OF BIO-RAD AG 50W AND DOWEX 50W IN LIQUID CHROMATOGRAPHY

UV filters: **, 260 nm; ***, 273 nm; ****, 280 nm; *****, 287 nm. Column: 18.5 × 0.8 cm; A = Bio-Rad AG 50W X8, Na^+ form (particle diameter 28 μm). Flow rate, 0.36 ml/min; Temperature 60°. B = Dowex 50W X8, Na^+ form (particle diameter 60 μm). Flow rate, 0.45 ml/min; Temperature, 52.5°.

$X \cdot C_6H_4 \cdot Y$		Position of Y	UV filter	A			B		
X	Y			Sample amount (μmole)	K_D (ml/g)	HETP (mm)	Sample amount (μmole)	K_D (ml/g)	HETP (mm)
H	SO_3Na		**	1.00		9.42			
	COONa		****	2.55	0.688	1.94	2.37	0.561	2.25
	OH		***	2.22	4.98	0.319	44.1	6.46	1.39
				22.1	4.69	0.954			
	NO_2		*****	1.66	12.7	0.307	1.66	17.7	1.21
	CH_2OH		**	35.2	3.88	0.377	35.2	4.80	1.43
	$COCH_3$		****	4.49	8.63	0.717	4.49	12.9	1.68
	CHO[a]		****	2.45	7.79	0.406	3.77	11.2	1.33
COONa	CH_3	2	****	2.71	0.660	4.28			
		3	****	2.57	1.16	4.28			
		4	****	2.50	1.27	4.96			
	OH	2	****	0.478	0.0802	1.60			
	NO_2	3	****	0.0270	0.137	1.60	1.86	0.104	1.57
		4	****	0.145	0.0930	1.28	0.251	0.0280	1.54
CH_3	SO_3Na	4	**				4.00		1.29
	NO_2[a]	2	****	0.475	20.8	0.402			
		3	*****	0.363	26.0	0.465			
		4	*****	0.322	23.5	0.487			
OH	CH_3	2	***	4.54	7.99	0.441	22.7	9.33	2.73
		3	***	5.05	8.16	0.561	20.3	10.2	2.07
		4	***	4.50	8.38	0.439	17.9	11.1	1.95
	OH	2	***	1.12	4.42	0.222		3.54	
		3	***	0.976	3.78	0.250		3.49	
		4	***	1.20	3.40	0.329	72.8	4.25	
	NO_2[a]	2	***	0.558	6.74	0.445	1.50	8.54	1.58
		3	***	0.827	13.9	0.362	15.4	20.3	1.18
		4	***	0.863	11.1	0.539	9.49	12.4	1.51
	Cl[a]	2	****	4.88	11.7	0.520	19.5	7.02	
		3	****	5.93	17.2	0.486	17.9	14.3	
		4	****	4.48	16.8	0.588	17.9	14.9	
	Br[a]	2	****	2.37	17.1	0.506			
		3	****	2.57	24.0	0.716			
		4	****	2.06	25.5	0.571			

[a] Column 6.7 × 0.8 cm.

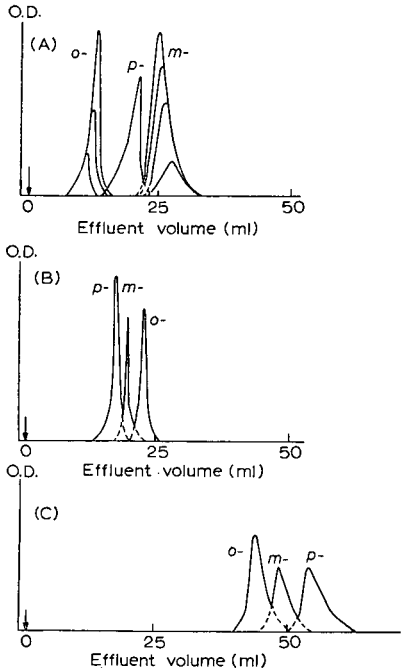

Fig. 6. Liquid chromatograms of phenols. Bio-Rad AG 50W X8. Diameter of particles, 28.1 μm (in H^+ form). Column diameter, 0.8 cm. Eluent, water. Flow rate, 0.36 ml/min. Temperature, 60°. (A) Nitrophenols: *ortho*, 0.56, 0.28 and 0.16 μmole; *meta*, 0.83, 0.65, 0.42 and 0.21 μmole; *para*, 0.18 μmole. Column length, 6.7 cm, Na^+ form. (B) Hydroxyphenols, *ortho*, 1.1 μmole; *meta* 0.98 μmole; *para* 1.2 μmole. Column length, 18.5 cm, Na^+ form. (C) Isopropylphenols, *ortho*, 1.67 μmole; *meta*, 1.69 μmole; *para*, 120. μmole. Column length: 18.5 cm, H^+ form.

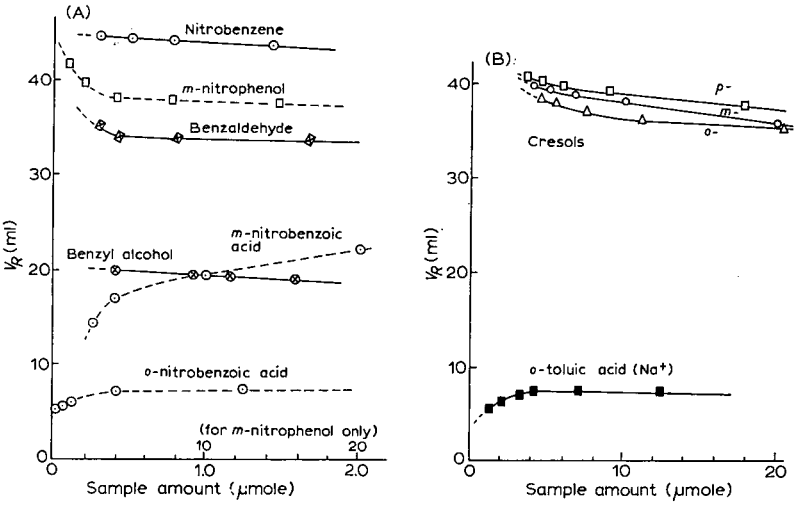

Fig. 7. Variation of V_R values of some benzene derivatives with change of solute concentration. (A) Dowex 50W X4. Mean diameter of particles, 65 μm. Column, 18.5 × 0.8 cm. Eluent, water. Flow rate, 0.45 ml/min. Temperature, 52.5°. (———), Na^+ form; (– – – –), H^+ form. (B) Bio-Rad AG 50W X 8. Mean diameter of particles; 28.1 μm (in H^+ form). Flow rate, 0.36 ml/min. Temperature, 60°. (———), Na^+ form.

was therefore increased by dilution of the solutes, but decreased by an increase in concentration.

As another example, the separation of *o*-, *m*- and *p*-dihydroxybenzene shows an almost linear adsorption isotherm (Fig. 6B). Chromatograms of *o*-, *m*- and *p*-isopropylphenol showed tailing and their complete separation was not achieved with the 18.5-cm column length (Fig. 6C). However, the separation of *o*- and *p*-isopropylphenol was complete.

The relation between the chemical structure of solutes and their K_D values will be discussed in a future paper.

CONCLUSIONS

The adsorption of benzene derivatives on the hydrogen and sodium forms of styrene-based strong cation-exchange resins was examined in aqueous solutions.

Hitherto, the character of non-ionic adsorption of aromatic compounds on strong cation-exchange resins was not certain. The present results explain some aspects of non-ionic adsorption. Thus there are some cases of linear adsorption isotherms and some cases of non-linear adsorption isotherms for which the variation of V_R value with solute concentration is not large. In these cases, a linear relation between peak heights or peak areas and solute concentration was obtained and quantitative analyses were possible.

Based on these results, some examples of possible separations were given, and K_D and HETP values, important data for the practical design of quantitative and qualitative analysis of benzene derivatives by liquid chromatography, were obtained for thirty-eight compounds.

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REFERENCES

1 N. NOMURA, D. SHIHO, K. OHSUGA AND M. YAMADA, *J. Chromatogr.*, 42 (1969) 226.
2 C. W. DAVIES AND G. G. THOMAS, *J. Chem. Soc.*, (1951) 2624.
3 O. SAMUELSON, *IVA*, 26 (1955) 178–188; *C.A.*, 50 1956) 4583a.
4 R. SARGENT AND W. RIEMAN, *J. Phys. Chem.*, 61 (1957) 354.
5 W. FUNASAKA, T. KOJIMA AND K. FUJIMURA, *Bunseki Kagaku*, 9 (1960) 852; 10 (1961) 374; 11 (1962) 936; 14 (1965) 820; 17 (1968) 48.
6 W. FUNASAKA, T. KOJIMA, K. FUJIMURA AND T. KUSHIDA, *Bunseki Kagaku*, 12 (1963) 466; 15 (1966) 835.
7 D. E. WEISS, *Nature*, 166 (1950) 66.
8 W. FUNASAKA, T. ANDO, K. FUJIMURA AND T. HANAI, *23rd Annual Meeting of the Chemical Society of Japan, Tokyo, April 4th, 1970.*
9 J. S. FRITZ AND A. TATEDA, *Anal. Chem.*, 40 (1968) 2115.
10 G. A. HARLOW AND D. H. MORMAN, *Anal. Chem.*, 36 (1964) 2438.
11 L. R. SNYDER, *Principles of Adsorption Chromatography; The Separation of Non-ionic Organic Compounds*, Marcel Dekker, New York, 1968.

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STUDIES ON LIGAND-EXCHANGE CHROMATOGRAPHY

III. SEPARATION OF NITROSONAPHTHOL ISOMERS

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SUMMARY

The separation of α-nitroso-β- and β-nitroso-α-naphthols by ligand-exchange chromatography was studied. The best results were obtained by use of a strong acid type resin in the Fe^{3+} form as a stationary phase and a 50 % v/v ethanolic ammonia solution (pH 9.5 and 12.0) as the mobile phase for stepwise elution. In view of the slow adsorption rate of both isomers to the resin, the Fe(III)–nitrosonaphthol complex formed in the resin phase seems to be a low-spin 1:1 complex for which the oxygen of the hydroxyl group and the nitrogen of the nitroso group are both responsible.

INTRODUCTION

The separation of isomers, homologues or analogues of organic substances from each other is generally difficult because of the similarity of their physico-chemical properties. If the substances to be separated are capable of coordinating with a metal ion, however, ligand-exchange chromatography using a cation exchanger or chelate resin in a metallic form, as a stationary phase, is very promising. In recent years, many studies on ligand exchange have been reported by several groups of workers[1–5], but the separation of isomers has rarely been tried.

In the previous work of this series[6,7], it was found that phenylenediamine isomers[6] could be completely separated on a column of Amberlite CG-120 in the Fe^{3+} form by eluting with very dilute aqueous ammonia (5×10^{-3} *M*) at room temperature. Similarly, aminobenzoic acid isomers[7] were separated on a column of the same resin in the Cu^{2+} form on eluting with either aqueous ammonia at pH 8.4 or distilled water alone. It was also found that dilute sodium hydroxide solution could be used as a developer with no metal leakage.

In order to apply the ligand-exchange reaction to elution chromatography, no metal leakage from the support and a fast rate of adsorption must be assured in order to achieve a successful separation. The former requirement may be satisfied to some extent by employing a resin which has a functional group that can form a complex with metal ions, *e.g.* a carboxylic acid type or iminodiacetic acid type resin, or by employing a developer which is as dilute as possible. On the other hand, to satisfy the second requirement, a metal ion which forms a labile complex, not neces-

sarily a complex of larger formation constant, with the substances to be separated must be selected as the counter ion of the resin.

The purpose of the present work is to find out the conditions appropriate for separating nitrosonaphthol isomers, to study the effect of alcohol on ligand-exchange adsorption, to determine the relationship between the rate of adsorption and the composition of the complex, the effect of physical adsorption on ligand-exchange adsorption, and the coordination site of the nitroso group.

EXPERIMENTAL

Reagents

α-Nitroso-β-naphthol and β-nitroso-α-naphthol: commercially available reagents (reagent grade; Tokyo Kasei Kogyo Co. Ltd.) were purified by recrystallization from 50 % v/v ethanol until the melting points agreed with the valves in the literature. Stock solutions were prepared by dissolving 0.1732 g of these substances in 230 ml of 50 % v/v ethanol and diluting to 250 ml with water; they were then stored in the dark. The concentration of this solution corresponds to 4×10^{-3} *M* (0.6928 mg/ml). Working standard solutions, whose concentrations ranged from 5×10^{-4} to 2×10^{-3} *M*, were prepared from the stock solution.

All the other reagents used were of analytical grade purity.

Ion-exchange resin: A strong acid cation-exchange resin, Amberlite CG-120, was used. After conditioning in the usual way, the resin was converted to the Fe^{3+} form with ferric chloride solution and was air-dried at room temperature by spreading on filter paper. The resin of a 100–200 mesh grade was used for batch operation and that of a 200–400 mesh grade for column operation.

Apparatus

A Shimadzu QV-50 spectrophotometer and a Toa Denpa HM-5A pH meter were used for spectrophotometric determination of the nitrosonaphthols and for the pH measurements, respectively. A RadiRac 3410B fraction collector (siphon type) was used for collecting the column effluents.

Distribution coefficients

The distribution coefficients for α-nitroso-β-naphthol and β-nitroso-α-naphthol on the Fe^{3+} form of the resin were measured by batch operation at room temperature as a function of the concentration of aqueous ammonia. To 1 g samples of the resin which had been weighed into 50-ml conical flasks with a glass stopper, 25 ml of aqueous ammonia of varying concentration was added. The flasks were then shaken gently for 1 h in order to swell the resin after which 1 ml of a nitrosonaphthol solution, corresponding to 0.1732 mg (1 μmole) of nitrosonaphthol, was added to the flasks. After being shaken vigorously for 1 h in a mechanical shaker, the flasks were allowed to stand for 24 h for equilibration to take place and then the resin was separated from the aqueous phase by filtration using a sintered-glass filter without suction. The amount of nitrosonaphthol in the solution was determined spectrophotometrically.

The distribution coefficient, K_d, was calculated as follows:

$$K_d = \frac{\text{mg of substance in resin phase/g of dry resin}}{\text{mg of substance in solution phase/ml of solution}}$$

The pH values of each equilibrated solution were also recorded.

Analysis

The nitrosonaphthols were determined by UV spectrophotometry using a quartz cell of 1.00 cm path length. As the absorption spectra of the nitrosonaphthols varied according to the pH value of the solution, the absorbances were measured at the isosbestic points, *i.e.* 280.0 nm for α-nitroso-β-naphthol and 294.0 nm for β-nitroso-α-naphthol. The relationship between pH value and absorptivity (extinction coefficient) at 280.0 and 294.0 nm is shown in Fig. 1. The leakage of ferric ion into the column effluent was tested by evaporating 500 ml portions of effluent to 50 ml and determining the ferric ion colorimetrically as the thiocyanate complex.

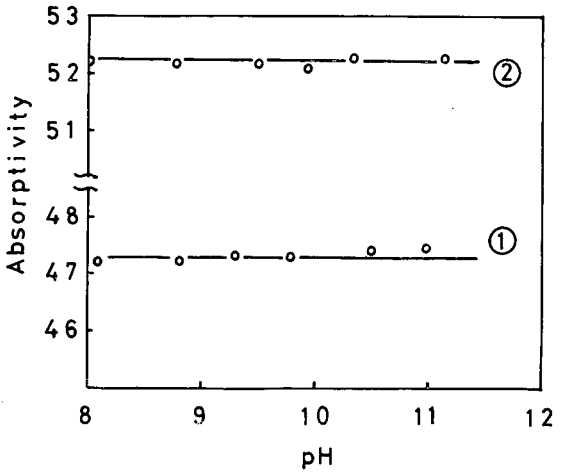


Fig. 1. Relationship between pH and absorptivity. (1) α-Nitroso-β-naphthol at 280.0 nm, concn. = 6.928 μg/ml; (2) β-nitroso-α-naphthol at 294.0 nm, concn. = 6.928 μg/ml.

Preparation of the column

Two or more volumes of dry resin (200–400 mesh, Fe^{3+} form) per volume of the chromatographic tube were placed in a large beaker. An appropriate volume of water or 50 % ethanol was poured into the beaker and the resin was stirred with a magnetic stirrer. After the resin had been swollen, concentrated aqueous ammonia was added dropwise using a pipet and the pH value of the solution was adjusted to the required value using a pH meter. After complete equilibration between the resin and the solution phases had been attained, the resin was separated from the solution by decantation and poured into a glass chromatographyc tube (11 mm diam. × 140 mm long) fitted with a stopcock. The equilibrated solution obtained by the above procedure was used as the developing solution.

In general, the resin used in the ligand-exchange technique must previously be equilibrated with the developer, because, otherwise, some water molecules coordinated with the ferric ion in the resin phase would be displaced by ammonia molecules or hydroxyl ions coming from the aqueous ammonia, and so the addition of a few drops of concentrated ammonia would cause little or no change in the pH value of the solution. It would be time-consuming to pack the column with resin that has been swollen in water alone and to equilibrate the resin by washing with a developer prepared independently.

RESULTS AND DISCUSSION

Effect of the ammonia concentration on adsorption

The distribution coefficients of α-nitroso-β- and β-nitroso-α-naphthols in aqueous ammonia on Amberlite CG-120 in the Fe^{3+} form are shown in Fig. 2 as a function of the pH value of the solution.

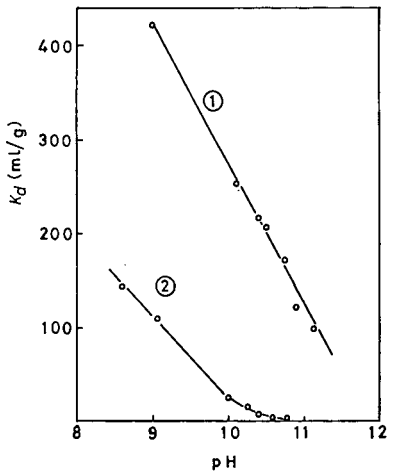


Fig. 2. Effect of pH (ammonia concentration) on the distribution coefficients of nitrosonaphthols. (1) α-Nitroso-β-naphthol; (2) β-nitroso-α-naphthol; resin: Amberlite CG-120, Fe^{3+} form.

It is obvious that the K_d values of these two substances, especially that of α-nitroso-β-naphthol, are quite large in neutral or very dilute ammonia solution and that they decrease with increasing pH value. The separation factor is large enough to permit a quantitative separation of the isomers in the pH range 8.0 to 11.0. The results shown in Fig. 2 also suggest that the formation constant of the Fe(III)–α-nitroso-β-naphthol complex is greater than that of the β-nitroso-α-naphthol complex.

Furthermore, the results are in good agreement with the generalization for ligand exchange that the ligands of higher coordination valency have a higher affinity to the metal ion in the resin phase than those of lower coordination valency, if the concentrations of ligands such as OH^-, NH_3 (monodentate) or nitrosonaphthol (bidentate) are low.

Effect of the ionic form of the resin on adsorption

The distribution coefficients of the nitrosonaphthols between resin in the Cu^{2+} form and aqueous ammonia are shown in Table I. Although the relationship between K_d and pH was nearly the same as that found for the resin in the Fe^{3+} form, the selectivity for nitrosonaphthol isomers followed the order β-nitroso-α- > α-nitroso-β-naphthol. This order was the reverse of that found for the resin in the Fe^{3+} form. The separation factor was too low to permit separation of the isomers by using the resin in the Cu^{2+} form.

Effect of ethanol on adsorption

In order to study the effect of ethanol on the adsorption of nitrosonaphthols,

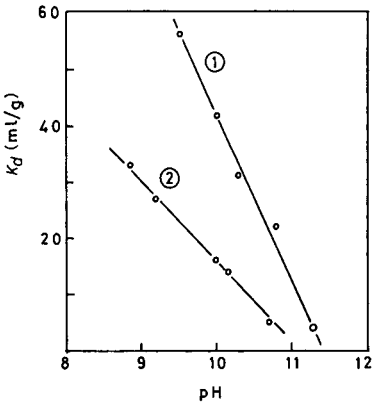
TABLE I

K_d VALUES OF NITROSONAPHTHOLS ON THE RESIN IN THE Cu^{2+} FORM AT VARIOUS pH VALUES

Substance	*pH value*[a]				
	9.0	*9.5*	*10.0*	*10.5*	*11.0*
α-Nitroso-β-naphthol	160.9	105.2	59.0	38.5	26.4
β-Nitroso-α-naphthol	203.3	126.1	73.9	48.2	38.1

[a] pH values were adjusted with aqueous ammonia.

the distribution coefficients were measured in an ethanolic ammonia solution. As can be seen in Fig. 3, the tendency of the results was similar to that observed in Fig. 2 when a 50 % v/v ethanolic ammonia solution was used instead of aqueous ammonia, but the K_d values and the separation factors were both much lower. This is probably due to the greater solubility of nitrosonaphthols in an ethanolic solution, rather than to the complex formation of ethanol with the ferric ion, since the absorption spectra of aqueous and 50 % ethanolic solutions of ferric ion (4×10^{-5} *M*), adjusted to pH 9.5 were identical in the range from 210.0 to 400.0 nm.



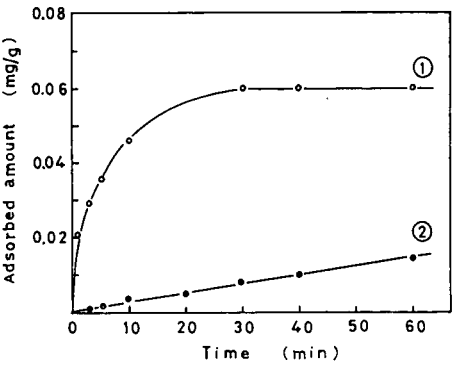


Fig. 3. Effect of ethanol on the distribution coefficients of nitrosonaphthols. (1) α-Nitroso-β-naphthol; (2) β-nitroso-α-naphthol. Resin: Amberlite CG-120, Fe^{3+} form; solvent: 50 % ethanolic ammonia solution.

Fig. 4. Rate of adsorption of nitrosonaphthols in aqueous ammonia at 23°. (1) α-Nitroso-β-naphthol; (2) β-nitroso-α-naphthol. Resin: Amberlite CG-120, Fe^{3+} form; solvent: aqueous ammonia (pH 9.6).

Rate of adsorption

The results of an examination of the sorption equilibria between nitrosonaphthols and the resin in the Fe^{3+} form are shown in Fig. 4: the amount of α-nitroso-β-naphthol adsorbed reaches its maximum within 30 min in aqueous ammonia at pH 9.6 and 23°, but that of β-nitroso-α-naphthol still tends to increase even after 60 min or more. These facts show that the rate of adsorption of α-nitroso-β-naphthol is comparatively slow, and that of β-nitroso-α-naphthol is even slower.

In general, a successful separation by ligand exchange requires not only a large

difference in the formation constants of the ligand complexes to be separated, but also a fast rate of equilibration. In order to carry out elution under equilibrium conditions when separating nitrosonaphthol isomers, the flow rate of the developer must be kept as slow as possible, within the limits between which no appreciable peak broadening occurs, owing to diffusion.

According to TAUBE[8], a d^5 metal ion, like Fe^{3+}, can form a high-spin labile complex as well as a low-spin inert complex. In view of the slow adsorption rate of nitrosonaphthols, the Fe(III)–nitrosonaphthol complex is probably a low-spin inert complex, containing OH^-, H_2O, NH_3 and nitrosonaphthol as its ligands.

Although the cause of the difference in adsorption rates between α-nitroso-β- and β-nitroso-α-naphthols is not quite clear, it can probably be attributed to the difference of the π-electron density of the nitroso group at the α- and β-positions: the electron density of a nitroso group in the β-position is higher than that in the α-position, so that it produces a stronger crystal field splitting.

Separation of isomers

From Figs. 2 and 3, it may be expected that the separation of the isomeric nitrosonaphthols would best be effected by stepwise elution using 50 % v/v ethanolic ammonia at different pH values, since their K_d values in aqueous ammonia are too large for their elution at a practical rate, even if a shorter column is employed.

When a mixture of these two isomers was eluted through a column (11 × 150 mm) using a 50 % v/v ethanolic ammonia solution, pH 9.5, at a flow rate of 0.25 ml/min, α-nitroso-β-naphthol was quantitatively retained on the column, and could be easily separated from β-nitroso-α-naphthol. After the complete elution of β-nitroso-α-naphthol with 100 ml of the above developer, the pH value of the developer was changed to 12.0 to elute the α-nitroso-β-naphthol rapidly. A typical elution curve is shown in Fig. 5. Both peaks show extreme tailing because of the slow adsorption rate of nitrosonaphthols: they are eluted before the sorption equilibrium has been attained at each plate of the column. The smaller retention volume of β-nitroso-α-naphthol, as compared with the value to be expected from Fig. 3, may be explained on the same grounds. The average recovery for α-nitroso-β- and β-nitroso-

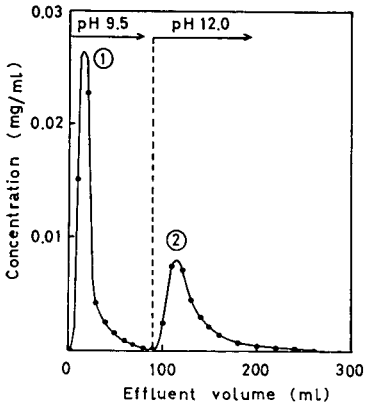


Fig. 5. Elution curve: (1) β-nitroso-α-naphthol; (2) α-nitroso-β-naphthol. Column size: 11 × 150 mm; developer: 50 % ethanolic ammonia solution; flow rate: 0.25 ml/min.

α-naphthol calculated from the results of three runs were 90.0 % and 96.0 %, respectively.

Composition of the complex

When three or more moles of nitrosonaphthol were present per mole of ferric ion in a weakly acidic or neutral solution, a water-insoluble black complex was formed from either nitrosonaphthol. In both cases, the composition of the complex, *i.e.* the molar ratio of ferric ion to nitrosonaphthol, was found to be 1:3 by the following method: the unaltered species were removed by treatment with 50 % ethanol, the complex was dissolved in absolute ethanol, conc. hydrochloric acid was added, and then ferric ion and nitrosonaphthol were determined by means of the thiocyanate method and UV spectrophotometry, respectively. The results are shown in Table II.

When the molar ratio of ferric ion to nitrosonaphthol was 1:1 or 1:2, no water-insoluble complex was formed, but the UV absorption spectra of the solution, shown

TABLE II

COMPOSITION OF Fe(III)-NITROSONAPHTHOL COMPLEXES (WATER-INSOLUBLE)

Complex	*Sample No.*	*Amounts in 50 ml of decomposed solution*		*Composition Fe(III) : ligand*
		Ferric ion[a] *(μmole)*	*Ligand*[b] *(μmole)*	
α-Nitroso-β-naphthol complex	1	0.42	1.21	1:2.92
	2	0.65	1.67	1:2.73
β-Nitroso-α-naphthol complex	1	0.70	1.96	1:2.80
	2	0.62	1.97	1:3.18

[a] Determined by the thiocyanate method.
[b] Determined by UV spectrophotometry.

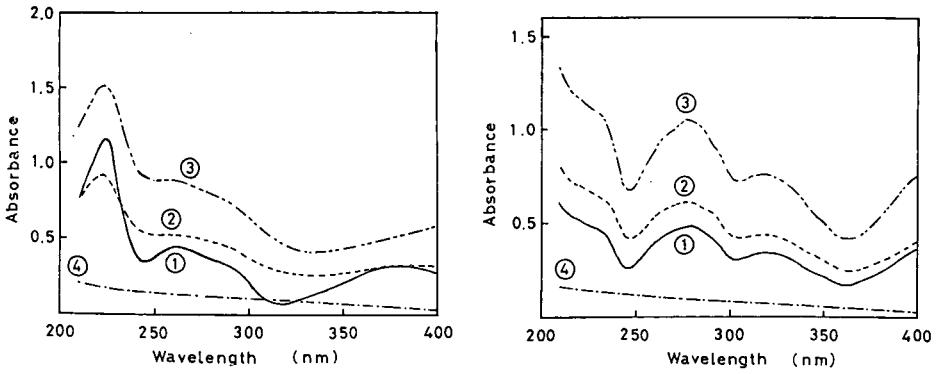


Fig. 6. Absorption spectra of Fe(III)–α-nitroso-β-naphthol complex. (1) α-Nitroso-β-naphthol (4×10^{-5} *M*); (2) Fe(III) (4×10^{-5} *M*) + ligand (4×10^{-5} *M*); (3) Fe(III) (4×10^{-5} *M*) + ligand (8×10^{-5} *M*); (4) Fe(III) (4×10^{-5} *M*).

Fig. 7. Absorption spectra of Fe(III)–β-nitroso-α-naphthol complex. (1) β-Nitroso-α-naphthol (4×10^{-5} *M*); (2) Fe(III) (4×10^{-5} *M*) + ligand (4×10^{-5} *M*); (3) Fe(III) (4×10^{-5} *M*) + ligand (8×10^{-5} *M*); (4) Fe(III) (4×10^{-5} *M*).

in Figs. 6 and 7, strongly suggest that some water-soluble complex or complexes are formed in solution.

In the ligand-exchange reaction, the ferric ions loaded on to the Amberlite CG-120, which had been equilibrated with aqueous ammonia at pH 9–10, must have formed either hydroxo–aquo or hydroxo–aquo–ammine mixed complexes: the IR spectrum of the resin equilibrated with aqueous ammonia at pH 9.5 was distinctly different from that equilibrated with sodium hydroxide solution at the same pH, especially at 3300–2800 cm^{-1} and at 1500–1400 cm^{-1}, suggesting the presence of the latter complex in the resin phase. Although an accurate composition of the complex formed in the resin phase is not known, it is evident that more than two moles of hydroxyl ion cannot have coordinated with one mole of ferric ion: the coordination of more than two moles of hydroxyl ion would make the complex neutral or negatively-charged and cause leakage of the ferric ion into the external solution, which in fact was not observed in the equilibrated solution. When a nitrosonaphthol solution is added to the resin under these conditions, one or two moles of the ligands that have coordinated with ferric ion will be displaced by nitrosonaphthol by ligand-exchange reaction: more than two moles of nitrosonaphthol cannot be adsorbed, because nitrosonaphthol acts as a univalent anion in alkaline solution and in addition the quantity is very small (1 μmole) compared with the amount of ferric ion in the resin phase. For the same reason, the sum of nitrosonaphthol and hydroxyl ions should never exceed two moles. Instead of a water-insoluble 1:3 complex, a 1:1 soluble complex might possibly be formed in the resin phase as well as in solution.

Effect of physical adsorption on ligand-exchange adsorption

It is known that most water-soluble aromatic compounds are adsorbed on ion-exchange resin by van der Waals' interaction between the solute and the resin matrix. In order to study the effect of any such adsorption which might occur during ligand-exchange adsorption, the retention volumes of the nitrosonaphthols were measured using a resin in the cationic form which had no coordination sites. The NH_4^+ form of Amberlite CG-120, 200–400 mesh, was chosen to avoid an ion-exchange reaction occurring between a cation loaded previously on to the cation exchanger and the ammonium ion in the external solution. The eluting conditions, such as column length, flow rate of developer, etc., were the same as those used in ligand-exchange chromatography.

As can be seen from Fig. 8, the retention volumes of two nitrosonaphthols, especially of β-nitroso-α-naphthol, were evidently greater than the column hold-up volume (about 10 ml) when they were eluted with aqueous ammonia at pH 9.5. These results suggest that the effect of physical adsorption cannot be neglected in ligand-exchange adsorption of nitrosonaphthols.

Determination of the coordination site of the nitroso group

The nitroso group is potentially capable of coordinating with a metal ion through either its oxygen or its nitrogen atom. According to FEIGL[9], the bond between α-nitroso-β-naphthol and a metal ion can be regarded as being formed through the nitrogen atom to give a five-membered chelate ring instead of a six-membered ring through the oxygen atom. Studies of the electronic configuration of the Co(II) complex also suggest that the bond is favoured through nitrogen, rather than oxygen[10].

On the other hand, CHATTERJEE[11] has reported that, in the case of the Cu(II) chelate of α-nitroso-β-naphthol, the bond is formed through the oxygen atom, in agreement with the generalization that a six-membered ring is more stable than a five-membered one when two double bonds are present in a chelate ring.

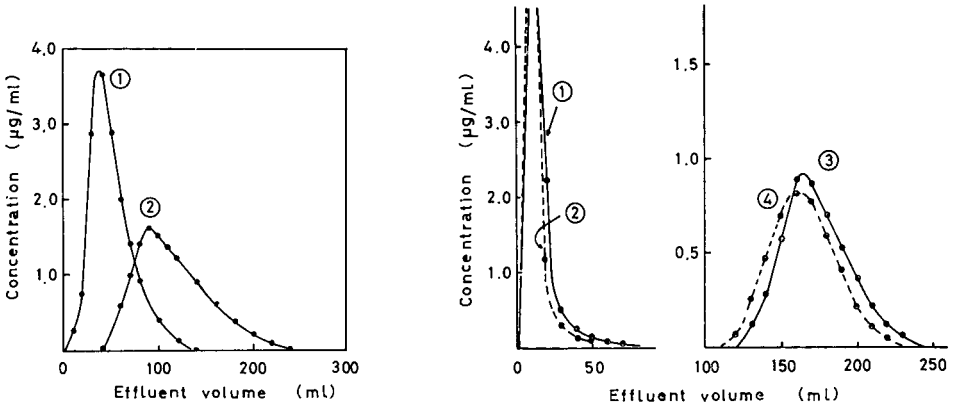


Fig. 8. Effect of physical adsorption on ligand-exchange adsorption. (1) α-Nitroso-β-naphthol; (2) β-nitroso-α-naphthol. Resin: Amberlite CG-120, NH_4^+ form; column size: 11 × 150 mm; developer: 50% ethanolic ammonia solution (pH 9.5); flow rate: 0.25 ml/min.

Fig. 9. Elution curves of hydroxynaphthoic acid and aminonaphthol. (1) 1-Hydroxy-2-naphthoic acid; (2) 2-hydroxy-1-naphthoic acid; (3) 1-amino-2-naphthol; (4) 2-amino-1-naphthol. Resin: Amberlite CG-120, Fe^{3+} form; column size: 11 × 150 mm; developer: aqueous ammonia (pH 9.5); flow rate: 0.25 ml/min.

In the present study, an attempt to determine the coordination site of the nitroso group was made by comparing the retention volume of hydroxynaphthoic acid and aminonaphthol with that of nitrosonaphthol. If the Fe(III) complex of nitrosonaphthol is formed through the oxygen atom in the nitroso group, the retention volume of nitrosonaphthol on the resin in the Fe^{3+} form would be similar to that of hydroxynaphthoic acid. On the other hand, if the complex is formed through the nitrogen atom, the retention volumes of nitrosonaphthol and aminonaphthol would be nearly the same. The retention volumes of 1-hydroxy-2- and 2-hydroxy-1-naphthoic acids and of 1-amino-2- and 2-amino-1-naphthols were measured, using a column of the same length as that used in ligand-exchange chromatography and are shown in Fig. 9: the hydroxynaphthoic acids were not adsorbed, whereas the aminonaphthols had a relatively large retention volume.

These results suggest that the Fe(III) complex of nitrosonaphthol is formed through the oxygen atom in the hydroxyl group and through the nitrogen atom in the nitroso group, to give a five-membered chelate ring. This view is also supported by the low-spin nature of the Fe(III) complex of nitrosonaphthol discussed above: the nitrogen atom having one pair of free electrons tends to produce a much stronger splitting of the crystal field of Fe(III) by concentrating the negative charge toward an e_g orbital of Fe(III), than the oxygen atom which has two of them.

ACKNOWLEDGEMENTS

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REFERENCES

1 K. SHIMOMURA, L. DIEKSON AND H. F. WALTON, *Anal. Chim. Acta*, 37 (1967) 102.
2 R. BOCK AND A. MONERJAN, *Z. Anal. Chem.*, 230 (1967) 1.
3 K. SHIMOMURA AND H. F. WALTON, *Separ. Sci.*, 3 (1968) 493.
4 C. A. BURTIS AND G. GOLDSTEIN, *Anal. Biochem.*, 23 (1968) 502.
5 R. A. A. MUZZARELLI, A. F. MARTELLI AND O. TUBERTINI, *Analyst*, 94 (1969) 616.
6 W. FUNASAKA, K. FUJIMURA AND S. KURIYAMA, *Jap. Anal.*, 18 (1969) 19.
7 W. FUNASAKA, K. FUJIMURA AND S. KURIYAMA, *Jap. Anal.*, 19 (1970) 104.
8 H. TAUBE, *Chem. Rev.*, 50 (1952) 69.
9 F. FEIGL, *Spot Tests in Inorganic Analysis*, 5th ed., Elsevier, Amsterdam, 1958, p. 144.
10 D. D. PERRIN, *Organic Complexing Reagents*, Interscience, New York, 1964, p. 217.
11 K. K. CHATTERJEE, *Anal. Chim. Acta*, 20 (1959) 423.

CHROM. 5390

DÜNNSCHICHT-SCRAPER — EIN HALBAUTOMATISCHES ABSCHABGERÄT FÜR FERTIGPLATTEN

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SUMMARY

Thin-layer scraper — a half-automatic scraper for precoated thin-layer plates

A new half-automatic scraper for precoated thin-layer plates is described. This scraper permits complete transfer of zones without mixing of test substances. The instrument is simple in operation. The zones are separated with high analytical accuracy in short time.

EINLEITUNG

Der Dünnschicht-Scraper (Fig. 1) wurde für die Wiedergewinnung organischer und anorganischer Substanzen in der Dünnschicht-Chromatographie entwickelt. Die Dünnschicht-Chromatographie zeichnet sich durch Schnelligkeit der Durchführung, hohe Trennschärfe sowie grosse Empfindlichkeit aus. Sie ist seit Jahren eine der vielseitigsten und meist benutzten Methoden zur analytischen und präparativen Trennung von Stoffgemischen.

Dünnschichtchromatographisch getrennte Substanzen müssen häufig zum Nachweis oder zu weiteren Verwendung vom Trägermaterial eluiert werden, was das Abschaben der substanzhaltigen Flecken oder Zonen erforderlich macht.

STAND DER TECHNIK

Nicht selten begegnet man in modern und zweckmässig eingerichteten Laboratorien umständlichen und ungenauen Abschabtechniken. Spatel, Rasierklingen oder Streifen eines Röntgenfilms bilden die ganze Abschabausrüstung. Die Einführung sogenannter "simple scrapers"[1], einfacher Abschabhilfen für einzelne Flecken oder Zonen, war zweifellos ein Fortschritt. Das gilt auch für sogenannte "Zonenkollektoren" oder "vacuum cleaners" (Mikrostaubsauger)[2], die jedoch nur zum Absaugen losen Trägermaterials geeignet sind. Diese Techniken sind nicht nur zeitraubend und oft ungenau, häufig geht beim Abtragen und Transfer durch den Verlust von Trägermaterial auch wertvolle Substanz verloren. Um diese Mängel beim Arbeiten mit radioaktiven Substanzen zu beheben, wurden von SNYDER und Mitarbei-

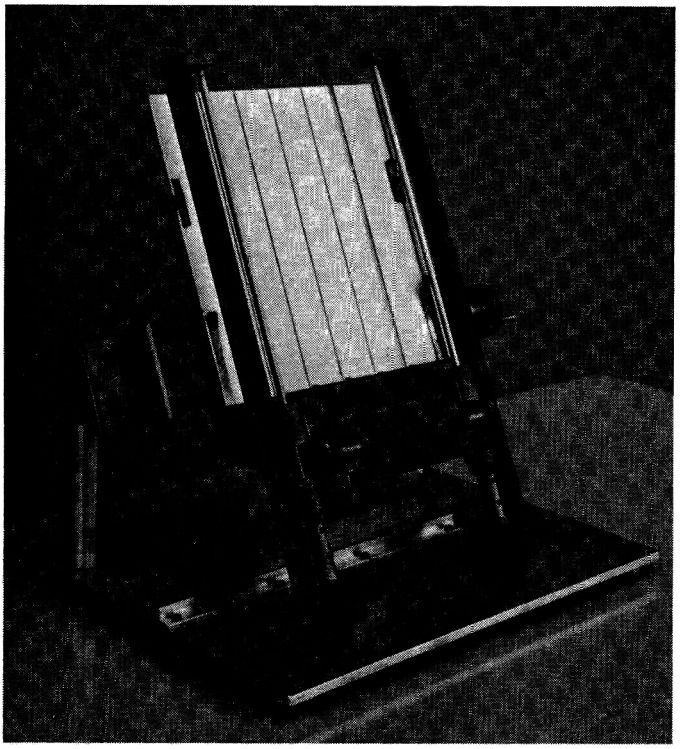

Fig. 1. Dünnschicht-Scraper. Messer 5 mm breit.

tern sogenannte "manual scrapers"[3] und "automatic zonal scrapers"[4,5] entwickelt. Alle "zonal scrapers" bestehen im wesentlichen aus einer Vorrichtung zur Befestigung und zum Vorschub der Platten, aus einem Schaber und einer Auffangvorrichtung für das abgeschabte Material. Ihre Funktion sei kurz beschrieben. Die Dünnschichtplatte wird nach ihrer Befestigung an einem Schlitten millimeterweise so an einer auf und ab schwingenden Schaberklinge vorbeigeführt, dass das abgeschabte Trägermaterial direkt in das Auffanggefäss fällt. Nach jedem Abschabvorgang rückt die Dünnschichtplatte entweder um 1, 2 oder 5 mm weiter, das unter dem Schaber befindliche Auffanggefäss wird jedesmal durch ein neues ersetzt. Bei den vollautomatischen "zonal scrapers"[6,7] werden wie bei den automatischen alle beweglichen Teile maschinell angetrieben, sie sind ausserdem mit einem Fraktionssammler und einer Pipettiereinrichtung zur automatischen Füllung von Szintillationsfläschchen versehen.

Die ersten Versuche mit "automatic zonal scrapers" bereiteten grosse Schwierigkeiten. Die Schichten wurden entweder unvollständig abgeschabt, das Trägermaterial platzte unvorschriftmässig ab, oder das abgeschabte Material blieb zum Teil an der Platte und am Schaber hängen. Um dem zuletzt genannten Übel abzuhelfen, wurden alle automatischen "zonal scrapers" mit Vibratoren ausgerüstet. Mit dieser Zusatzeinrichtung kann man die Platte in Schwingungen versetzen und lose anhängendes Material vollständig abschütteln. Die "zonal scrapers" bewährten sich vorwiegend bei nicht zu losen, aber nicht bei zu festen Schichten. Lose Schichten werden bei

Verwendung von Vibratoren beschädigt, feste Schichten, wie sie z.B. bei den meisten handelsüblichen Fertigplatten vorliegen, pflegen bei maschineller Bearbeitung leicht zu reissen und abzuplatzen.

Mit dem von uns entwickelten Dünnschicht-Scraper können wir lose und feste Schichten, praktisch alle bekannten und handelsüblichen Dünnschichtplatten und -folien ohne Verlust abschaben.

Der Dünnschicht-Scraper ist von der Garching Instrumente (München) und der Desaga (Heidelberg) für die industrielle Fertigung weiterentwickelt worden. Patent- und Gebrauchsmusterschutz wurden angemeldet.

BESCHREIBUNG DER KONSTRUKTION

Die Konstruktion des Dünnschicht-Scrapers ist aus der schematischen Darstellung (Fig. 2) zu ersehen. In dieser Zeichnung weist der an einem Stativ (1) befestigte, gegen die Vertikale um 25° geneigte Arbeitsschlitten (2) zwei oder vier Halterungsfedern (3) für die Dünnschichtplatte auf. Die mit einer Handkurbel (4) versehene Antriebsspindel (5) dient der horizontalen Schlittenfortbewegung. Der Feinantrieb ermöglicht eine Einstellgenauigkeit von ± 0.1 mm. Dieselbe Neigung wie der Arbeitsschlitten (2) haben zwei an Gleitschienenhalterungen (6) befestigte Vertikalgleitschienen (7), auf denen Kugelgleitbüchsen (8) laufen, die ihrerseits über Horizontalschwenkachsen (9) mit der Schaberplatte (10) verbunden sind. Auf dem Oberkantenteil der Schaberplatte (10) sind verstellbare Schaberhalter (11) angebracht, in die auswechselbare, in Form von flachen Klingen ausgebildete Schaber (12) in der Weise einsetzbar sind, dass sich die Klingenenden in einer Ebene befinden. Die Klingen sind scharf geschliffen und leicht nach unten gebogen. Sie bestehen aus sehr hartem und elastischem Material, z.B. aus Uhrfederstahl.

Unterhalb der Schaber sind auf einer Trichterleiste (13) Trichter angeordnet, durch die das abgeschabte Material in Probenauffanggefässe (14) geleitet wird, die

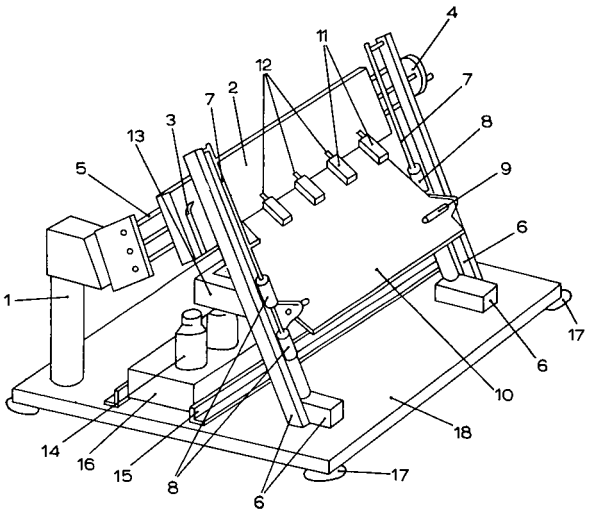

Fig. 2. Schematische Darstellung des Dünnschicht-Scrapers. Dünnschicht-Platte 200 mm lang, 50 mm breit; Messer 5 mm breit. Für Erläuterung siehe BESCHREIBUNG DER KONSTRUKTION.

in einem zwischen Horizontalgleitschienen (15) linear beweglichen Gefässschlitten (16) befestigt und unter die Trichter zu schieben sind. Das Stativ (1), die Gleitschienenhalterung (6) und die Horizontalgleitschienen (15) sind auf einer mit Sauggummis (17) versehenen Bodenplatte (18) montiert.

ANWENDUNG

Die Konstruktion des Dünnschicht-Scrapers ermöglicht in einem Arbeitsgang gleichzeitiges Abschaben von ein bis fünf Streifen. Die leichte Neigung des Arbeitsschlittens und die manuelle Beweglichkeit der Messerleiste bieten gegenüber dem "zonal scraper" die Möglichkeit, Schabgeschwindigkeit und Messerdruck dem Schichtmaterial anzupassen. Die Verwendung dieses Abschabgerätes führt zu zahlreichen wesentlichen Vorteilen: kein Vermischen von Zonen und Abplatzen von Trägermaterial, scharfe Streifen und feinkörnig abgeschabtes Material.

So können z.B. dünnste Zonen mit bisher nicht erreichter Präzision in Form eines pulverförmigen, leicht zu eluierenden Schichtträgermaterials abgetragen werden. Aber auch verhältnismässig dicke Schichten, z.B. solche von sogenannten präparativen Platten, lassen sich mit diesem Scraper unter Verwendung einer Schablone praktisch verlustlos aufarbeiten.

Mit Hilfe des Dünnschicht-Scrapers sind verschieden starke, z.B. 0.1 bis 2 mm dicke Schichten verschiedenen Typs abschabbar. Zu verwenden sind alle bekannten Dünnschichtplatten und -folien der Ausmasse 50 × 200, 100 × 200 oder 200 × 200 mm (Fig. 1). Es kann sich um sogenannte Fertigplatten oder um solche mit Handstreichgeräten selbst hergestellte Dünnschichtplatten handeln. Nach einer grossen Anzahl von Versuchen zeigte sich, dass bei Verwendung dieses Dünnschicht-Scrapers der Typ der Beschichtungsmasse ebensowenig eine Rolle spielt wie die Breite der verwendeten Schaberklinge. Das ist darauf zurückzuführen, dass man die Schaberplatte in einfacher Weise mit mehr oder weniger starkem Druck betätigen kann. Verglichen mit dem "manual zonal scraper" arbeitet man mit dem Dünnschicht-Scraper wesentlich schneller und sauberer. Im Vergleich zum automatischen "zonal scraper" dauert das Abschaben mit dem Dünnschicht-Scraper zwar etwas länger, dafür ist das Gerät wesentlich kleiner und billiger, ausserdem einfacher im Aufbau, in der Bedienung und Wartung. Der Dünnschicht-Scraper übertrifft die "zonal scrapers" an Vielseitigkeit, zuweilen auch an Genauigkeit und Zuverlässigkeit. Die Zeitersparnis beträgt bei der Anwendung des Dünnschicht-Scrapers im Vergleich zur einfachen Abschabmethode mit einem Spatel etwa 90 %.

BEDIENUNG

Nach der dünnschichtchromatographischen Trennung des Substanzgemisches in einzelne Zonen oder Flecken wird das Chromatogramm auf dem Arbeitsschlitten (2) mit Halterungsfedern (3) festgeklemmt. Durch Grobeinstellung kann man innerhalb kürzester Zeit die passenden 1 bis 10 mm breiten Klingen (12) des Schabers (10) auf die interessanten Bereiche einstellen, dann durch Feinantrieb mit der Handkurbel (4) die Klingen genau auf die abzuschabenden Zonen oder Flecken ausrichten. Die Trichter an der Trichterleiste (13) und die Probenauffanggefässe (14) im Gefässschlitten (16) lassen sich mit wenigen Handgriffen so anordnen, dass sie direkt unter den Schaberklingen bzw. Zonen stehen. Nach Einrichten von Platte und Gerät werden die Schaberklingen in der Weise von oben nach unten über die Dünnschicht gezogen,

dass anfangs flache, der Führung des Materials dienende Rillen entstehen. Durch Verstärkung des Drucks auf die Klingen und mehrmaliges Schaben lässt sich das Trägermaterial ohne vorheriges Markieren innerhalb kürzester Zeit praktisch vollständig in die betreffenden Probenauffanggefässe überführen.

Soll das gesamte Trägermaterial einer Platte in gleich breiten Streifen abgetragen werden, z.B. ein 150 mm langes Chromatogramm durch 6-maliges Abschaben mit fünf Klingen von je 5 mm Breite in 30 gleichen Fraktionen, geht man am besten so vor, dass man nach jedem Abschabvorgang die Klingen immer auf die jeweils benachbarten Streifen einstellt und diese sorgfältig abkratzt. Dabei ist es gleichgültig, ob man am Start oder an der Front des Chromatogramms beginnt. Die Gefässschlitten und Stellflächen der Probenauffanggefässe müssen stets numeriert werden, damit bei den Probenauffanggefässen und dem abgeschabten Material keine Verwechslungen entstehen.

Dünnschichtplatten und -folien der Formate 50 × 200 und 100 × 200 mm lassen sich verhältnismässig leicht abschaben, das gilt insbesondere für 0.1–0.5 mm dicke Schichten. Beim Format 200 × 200 mm und 0.5–2 mm dicken Schichten empfiehlt sich die Verwendung einer Schablone, die vor dem Abschaben auf die Platte gesetzt wird. Die Schlitze in der Schablone erleichtern zusätzlich die Führung und den Transport des abgeschabten Materials in die Probenauffanggefässe.

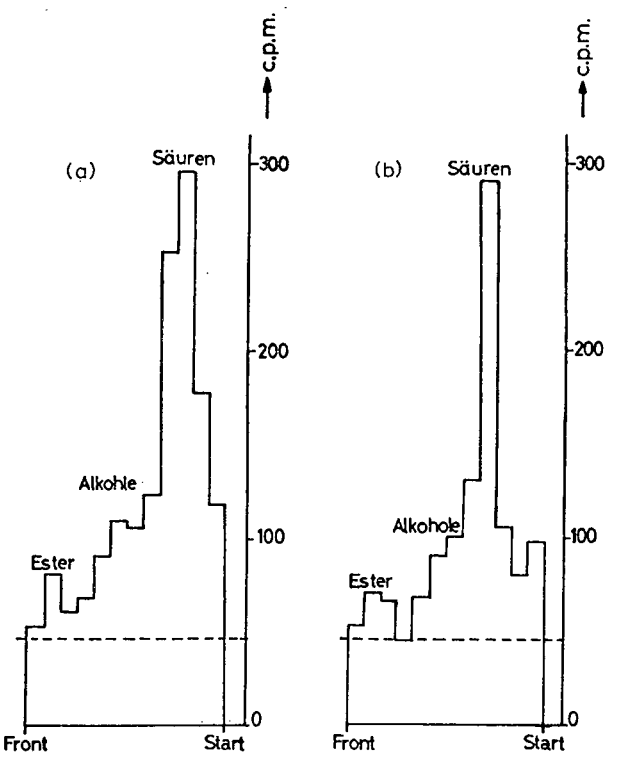

Fig. 3. Radiochromatogramme eines Lipidgemisches. Fliessmittel: Diisopropyläther. Manuell abgeschabt; Spatel 5 mm breit.

Die Trichterleiste, sowie die Schaber und Schaberplatte sind mit wenigen Handgriffen auswechselbar und leicht zu reinigen.

EXPERIMENTELLE ERGEBNISSE UND DISKUSSION

Die folgenden Beispiele sollen die Leistungsfähigkeit des Dünnschicht-Scrapers demonstrieren. Es wurden Tritiummarkierte Lipidgemische[8] hoher spezifischer Aktivität chromatographiert, die bei der Isolierung von Metaboliten des Sexuallockstoffes ^{3}H-Bombykol[9] anfallen. So gelingt es z.B. die radioaktiv markierten Metaboliten in Mengen von nur 10^{-5} bis 10^{-6} μg, entsprechend 10^9 bis 10^{10} Molekülen aus Antennen des Seidenspinners *Bombyx mori* L. zu isolieren, zu trennen und zu bestimmen. In Kombination mit einem Flüssig-Szintillations-Spektrometer übertrifft der Dünnschicht-Scraper an Empfindlichkeit und Genauigkeit bei weitem einen Dünnschicht-Scanner[10].

In allen Abbildungen ist auf der Ordinate die gemessene Tritium-Aktivität in c.p.m. oder d.p.m. (Impulse oder radioaktive Zerfälle pro Minute) aufgetragen, die Abzisse gibt die Laufstrecke und damit die Stelle der Platte wieder, von der die Probe abgeschabt worden war. Verwendet wurden 0.25 mm dicke Fertigplatten "Kieselgel H" der Firma Merck.

Als Vergleichsbeispiel zunächst das manuelle Abschaben eines mit Diisopropyläther chromatographierten radioaktiven Metabolitengemisches[8]. Die Chromatogramme wurden mit einem 5 mm breiten Spatel in Form von je zwölf Fraktionen innerhalb von 30 min aufgearbeitet. Trotz gleicher Lipidzusammensetzung weisen

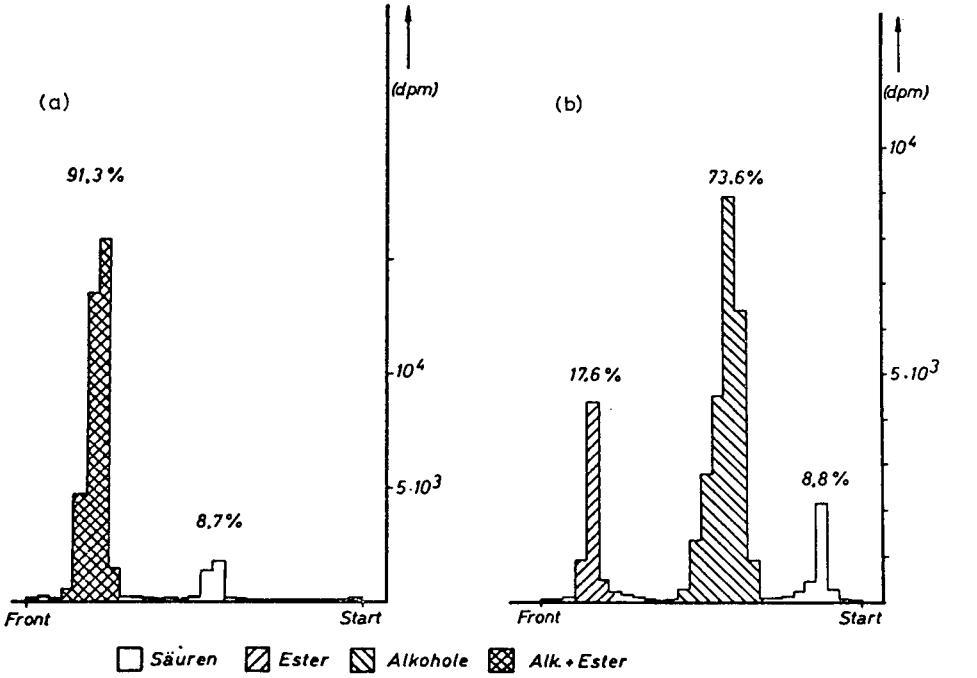

Fig. 4. Radiochromatogramme von Lipidgemisch IV (siehe Tabelle I). Dünnschicht-Scraper; Messer 5 mm breit. (a) Fliessmittel a; (b) Fliessmittel b.

beide Versuche keine Reproduzierbarkeit der Ergebnisse auf (Fig. 3). Den Zuwachs an Genauigkeit und Zeitgewinn demonstrieren folgende Versuche: die Lipidgemische I bis IV wurden entweder zweimal mit demselben oder je einmal mit zwei verschiedenen Fliessmitteln chromatographiert. Ein Chromatogramm ist innerhalb von 10 min mit dem Dünnschicht-Scraper vom Schichtträger abgetragen und für die Radioaktivitätsbestimmung im Flüssig-Szintillations-Spektrometer vorbereitet worden. Die Radiochromatogramme (Fig. 4) zeigen, dass die Lipidkomponenten nunmehr klar getrennt wurden, auch mit verschiedenen Fliessmitteln ergaben sich praktisch gleiche Zusammensetzungen (Tabelle I).

TABELLE I

ZUSAMMENSETZUNG TRITIUM-MARKIERTER METABOLITEN-GEMISCHE

Fertigplatte: Kieselgel H 0.25 mm der Firma Merck. Fliessmittel: (a) *n*-Butanol, gesättigt mit 12.5 prozentigem NH_3; (b) *n*-Hexan-Diisopropyläther–Methanol-12.5 prozentiges NH_3 (100:20:10:0.15). Dünnschicht-Scraper; Messer 5 mm breit. Flüssig-Szintillations-Spectrometer: Packard Tricarb, Modell 3380.

Lipid-Gemisch	*Fliess-mittel*	*Säuren* (%)	*Alkohole* (%)	*Ester* (%)	*Alkohole + Ester* (%)
I	b	8.2	86.6	5.2	91.8
		8.7	87.3	4.0	91.3
II	b	19.5	72.3	8.2	80.5
		18.7	72.3	9.0	81.3
III	b	21.3	66.6	12.1	78.7
	a	22.6	—	—	77.4
IV	b	8.8	73.6	17.6	91.2
	a	8.7	—	—	91.3

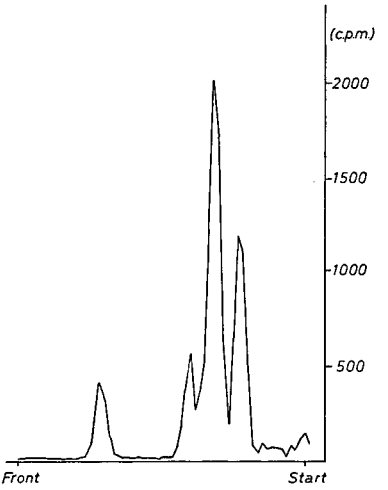

Fig. 5. Radiochromatogramm eines Lipidgemisches. Fliessmittel b (siehe Tabelle I). Dünnschicht-Scraper; Messer 2.5 mm breit.

Benützt man statt der 5-mm Klingen solche von 2.5 mm Breite, spaltet man also das abgetragene Material in doppelt so viele Fraktionen auf, steigt die Auflösung entsprechend. Im Experiment konnten bei ähnlichen Lipidgemischen sonst nicht zu trennende Substanzgemische in ihre Komponenten aufgespalten werden (Fig. 5).

ZUSAMMENFASSUNG

Es wird ein halbautomatischer Scraper für Fertigplatten beschrieben. Dieser Scraper gestattet vollständigen Transfer der Zonen ohne Vermischen der Testsubstanzen. Das Gerät ist einfach in der Bedienung, die Zonen werden mit hoher analytischer Genauigkeit in kurzer Zeit abgetrennt.

LITERATUR

1 S. BJÖRKERUD, *J. Lipid Res.*, 10 (1969) 459.
2 F. J. RITTER UND G. M. MEYER, *Nature*, 193 (1962) 941.
3 F. SNYDER, *Anal. Biochem.*, 9 (1964) 183.
4 F. SNYDER UND H. KIMBLE, *Anal. Biochem.*, 11 (1965) 510.
5 F. SNYDER, *Atomlight*, 58 (1967) 1.
6 F. SNYDER UND D. SMITH, *Separ. Sci.*, 1 (1966) 709.
7 F. SNYDER, *Current Status of Liquid Scintillation Counting, Proc. Symp. Cambridge, Mass., March 31–April 3, 1969*, Grune and Stratton, New York, 1969.
8 G. KASANG, *Int. Symp. Gustation and Olfaction, Geneva, 1970*, Academic Press, London, New York, 1971, S. 245–250.
9 G. KASANG, *Z. Naturforsch.*, 23b (1968) 1331.
10 P. E. SCHULZE UND M. WENZEL, *Angew. Chem.*, 74 (1962) 777.

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THE THEORETICAL STUDY OF THE PAPER ELECTROPHORETIC SEPARATION OF LOW MOLECULAR WEIGHT SUBSTANCES

I. APPLICATION TO AMINO ACIDS AND PEPTIDES

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SUMMARY

The relative zone mobility of substances with dissociable protons is expressed in terms of the molecular weight, the charge, the dissociation constant of the proton, and the pH of the background solution. Using the derived equation the separability of given mixtures is discussed.

Relative mobility against pH curves of amino acids and peptides were obtained using this equation. Many previously published results were compared with those calculated and were found to be in good agreement with the exception of a few amino acids. The differences between the observed and the calculated results is discussed based on the charge, the molecular weight, the chemical structure, and the degree of hydration of the substances under consideration.

Thus, if pK values and molecular weights of given substances are known, the optimum conditions for separation can be read off from calculated mobility–pH curves rather than obtained by laborious experimental tests.

INTRODUCTION

Paper electrophoresis is used mainly for the separation of various substances. As electrophoretic experiments are simple and usually fast this method soon became very popular in chemistry, biochemistry, and medicine[1,2]. Recently it has also been applied in the chemistry[3–6] of complexes and for the study of ion pair formation[7,8].

In spite of its frequent usage and the advances in techniques and apparatus, it is probable that no physico-chemical method has ever been used so extensively with so little knowledge of its fundamentals[9].

Because of the various parameters influencing the process the prospects of deriving a generally applicable theory are rather poor. The first approach to a theory was the application of the STOKES' law[10] and the adaptation of this law for the calculation of the mobilities of smaller size particles by changing the numerical factors of this law[11–13]. JOKL derived an equation for electrophoretic mobilities by studying the dependence of these mobilities on the mass of the species[14]. The mobilities in free solution and the decrease of the mobility on a paper strip due to an obstructive

factor[15–20] and an adsorptive factor[17–20] were investigated. CONSDEN *et al.* studied the mobilities of dissociating substances depending on their pK values[21]. Their equation could be applied to substances with only one dissociable proton.

Recently an equation for the mobility of substances with more than one dissociable proton, as a function of their molecular weight, the charge of the migrating species, their proton dissociation constant and the pH of the background solution, was derived[22].

In this paper we report detailed studies on this theory, its theoretical applicability in different cases, and experimental and calculated results are compared. We try to predict optimum conditions for separations with special emphasis on the study of amino acids and peptides.

THEORETICAL CONSIDERATIONS

The effective charge of molecules with intrinsic ionized groups such as organic acids, organic bases or amino acids depends on the pH of the medium and the pK(s) of the ionized group(s). Amphoteric molecules like amino acids (H_nA) form cations in an acidic solution by binding proton(s), while in a basic solution they form anions by dissociating proton(s). The degree of association or dissociation of the protons is a function of the pH of the background solution and the pK(s) of the ionized substance(s). The equilibrium relation between the proton dissociation of amino acids and the pH of the medium can be expressed as follows:

$$H_{m+n}A^m \overset{-H^+}{\rightleftharpoons} H_{m+n-1}A^{m-1} \ldots H_{m+n-(i-1)}A^{m-(i-1)} \overset{-H^+}{\rightleftharpoons} H_{m+n-i}A^{m-i}$$

$$\ldots HA^{-(n+1)} \overset{-H^+}{\rightleftharpoons} A^{-n} \qquad (1)$$

where m represents the maximum number of protons which can be bound by the imino or amino groups, n is the maximum number of dissociable protons which are due to –COOH, –OH, or –SH groups.

In this proton equilibrium system, eqn. 1, the proton-exchange reaction is reversible and the reaction rate is usually very high. Consequently the mean charge of all the ionic species in the equilibrium system can be expressed as follows:

$$Z = \frac{\sum_{i=0}^{m+n} (m-i)\,[H_{m+n-i}A^{m-i}]}{\sum_{i=0}^{m+n} [H_{m+n-i}A^{m-i}]} \qquad (2)$$

Eqn. 3. is the definition of the consecutive proton dissociation constant.

$$K_i = \frac{[H_{m+n-i}A^{m-i}]\,[H^+]}{[H_{m+n-(i-1)}A^{m-(i-1)}]} \qquad i = 1 \sim m+n \qquad (3)$$

By substitution of eqn. 3 into eqn. 2, we get

$$Z = \frac{m + (m-1)K_1/[H^+] + (m-2)K_1K_2/[H^+]^2 + \ldots +}{1 + K_1/[H^+] + K_1K_2/[H^+]^2 + \ldots +}$$

$$\frac{(m-i)K_1K_2 \ldots K_i/[H^+]^i + \ldots + (-n)K_1K_2 \ldots K_{m+n}/[H^+]^{m+n}}{K_1K_2 \ldots K_i/[H^+]^i + \ldots + K_1K_2 \ldots K_{m+n}/[H^+]^{m+n}} \quad (4)$$

As the calculation of the mean charge using eqn. 4 is complicated, we tried to simplify it.

If we choose only two successive ion species, such as $H_{m+n-(i-1)}A^{m-(i-1)}$ and $H_{m+n-i}A^{m-i}$ from all the species in the equilibrium system (1), the mean charge is given by

$$Z = \frac{(m-(i-1))\,[H_{m+n-(i-1)}A^{m-(i-1)}] + (m-i)\,[H_{m+n-i}A^{m-i}]}{[H_{m+n-(i-1)}A^{m-(i-1)}] + [H_{m+n-i}A^{m-i}]}$$

$$= (m-(i-1)) \cdot \frac{1 + \alpha_i K_i/[H^+]}{1 + K_i/[H^+]}$$

$$= (m-(i-1)) \cdot \left[\frac{1+\alpha_i}{2} + \frac{1-\alpha_i}{2} \cdot \frac{1 - K_i/[H^+]}{1 + K_i/[H^+]}\right]$$

$$= \frac{1}{2} + (m-i) + \frac{1}{2} \cdot \frac{1 - K_i/[H^+]}{1 + K_i/[H^+]} \quad (5)$$

where $\alpha_i = (m-i)/(m-(i-1))$
Now, if we define

$$K_i/[H^+] = e^{-2x} \quad (6)$$

we obtain

$$x = \frac{2.303}{2}(pK_i - pH) \quad (7)$$

Further, combining the well known relation

$$\frac{e^x - e^{-x}}{e^x + e^{-x}} = \frac{1 - e^{-2x}}{1 + e^{-2x}} = \tanh x \quad (8)$$

with eqns. 6 and 7, eqn. 5 is altered to eqn. 9:

$$Z_i = (m-(i-1)) \left[\frac{\alpha_i + 1}{2} + \frac{1-\alpha_i}{2} \tanh\left\{\frac{2.303}{2}(pK_i - pH)\right\}\right]$$

$$= \left[\left(m - i + \frac{1}{2}\right) + \frac{1}{2} \tanh\left\{\frac{2.303}{2}(pK_i - pH)\right\}\right] \quad (9)$$

If $i < m$, Z_i in eqn. 9 is positive. On the other hand, if $i > m$, Z_i is negative. The mean charge is represented schematically as a function of the pH in Fig. 1. The first term, $m - i + \frac{1}{2}$ (eqn. 9), corresponds to the distance between the inflection point of the sigmoidal curve and the pH-axis, the $\frac{1}{2}$ in the second term represents the amplitude of the curves, and pK_i the distance between the inflection point of the

curves and the Z axis, respectively. The mean charge Z in Fig. 1 only refers to two successive ionic species. The overall charge achieved by the presence of various ionic species as a function of the pH is shown in Fig. 2, the calculation being based on eqn. 4. An equation representing this continuous Z–pH curve is not the simple summation

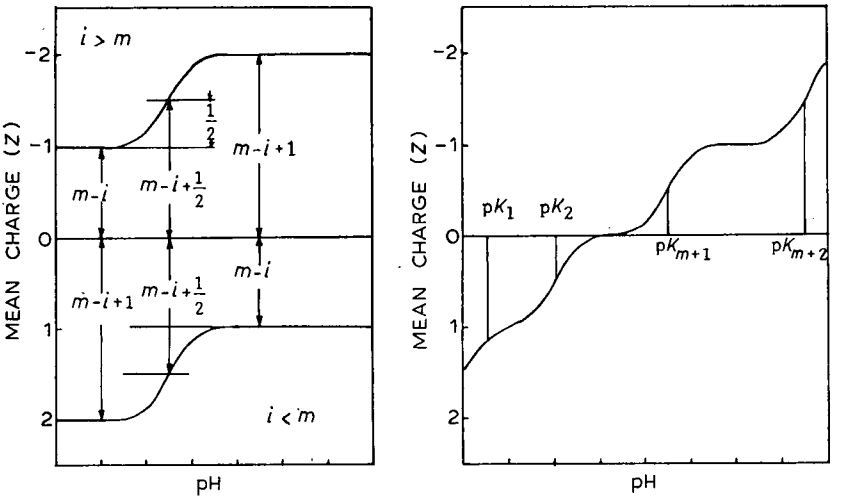

Fig. 1. pH dependence of the mean charge for proton dissociation and protonation equilibrium, respectively.

Fig. 2. pH dependence of the mean charge for more than one consecutive protonation or dissociation equilibrium.

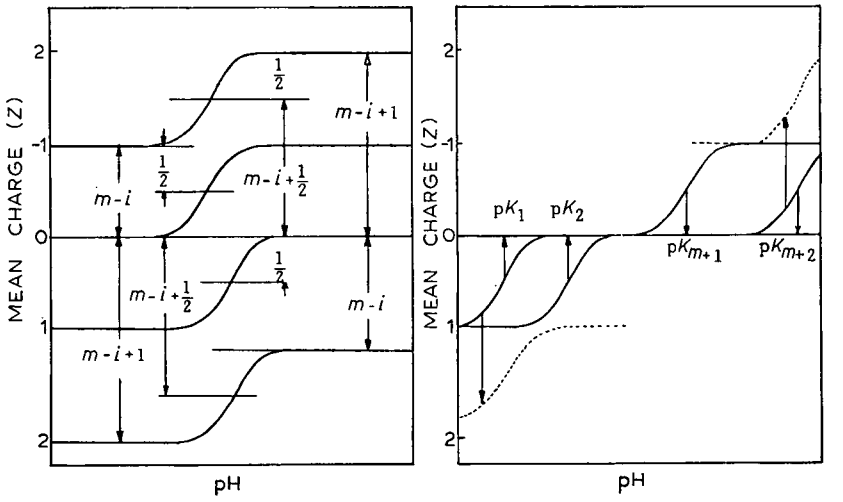

Fig. 3. pH dependence of the mean charge showing the modification of eqn. 9 to eqn. 10 graphically.

Fig. 4. pH dependence of the mean charge for various consecutive protonation or dissociation equilibria showing the modification of eqn. 9 to eqn. 10 schematically.

of eqn. 9 from $i = 1$ to $i = m + n$. In order to get a component function of the desired equation, the first term of eqn. 9 should be modified as follows:

$$Z_i = \frac{1}{2}\left[1 + \tanh\left\{\frac{2.303}{2}(pK_i - pH)\right\}\right] \tag{10}$$

The modification in eqn. 10 is due to the shift of the Z axis from $m - i$ to zero as shown in Fig. 3.

In general, the relationship between $pK_i = 1 \sim m + n$ is as follows:

$$pK_1 < pK_2 < \ldots pK_{i-1} < pK_i < \ldots < pK_{m+n} \tag{11}$$

The Z_i–pH curves obtained with eqns. 10 and 11 are drawn as solid lines in Fig. 4.

$$\begin{aligned} Z &= \sum_{i=1}^{m} Z_i + \sum_{m+1}^{m+n} Z_i \\ &= \frac{1}{2}\sum_{i=1}^{m}\left[1 + \tanh\left\{\frac{2.303}{2}(pK_i - pH)\right\}\right] \\ &\quad - \frac{1}{2}\sum_{i=m+1}^{m+n}\left[1 + \tanh\left\{\frac{2.303}{2}(pH - pK_i)\right\}\right] \end{aligned} \tag{12}$$

The modification of eqn. 9 to eqn. 10 may be understood from the schematic diagram in Fig. 4. As the charge of the ionic species in the proton equilibrium system (eqn. 1) is always positive in the range $i < m$, Z can be expressed by eqn. 13 which is derived from the first or the second term of eqn. 12 by adding or subtracting the shift of the coordinate Z, respectively.

$$\begin{aligned} Z &= \frac{1}{2}\sum_{i=1}^{m+n}\left(1 + \tanh\left\{\frac{2.303}{2}(pK_i - pH)\right\}\right) - n \\ Z &= m - \frac{1}{2}\sum_{i=1}^{m+n}\left[1 + \tanh\left\{\frac{2.303}{2}(pH - pK_i)\right\}\right] \end{aligned} \tag{13}$$

m in eqn. 13 is zero for organic acids whereas for substances like amines, purine and pyrimidine bases, n is zero.

THE RELATIONSHIP BETWEEN THE ZONE MOBILITY AND THE MASS OF MIGRATING SUBSTANCES

Recently a relationship between the zone mobility of the migrating substances and their molecular weight has been derived. It proved to be in good agreement with many experimental results[14, 22–24]

$$U = a\frac{Z}{\sqrt{M}} - b \tag{14}$$

where U is the relative zone mobility of the migrating species, M is the mass, *i.e.* molecular weight, a and b are constants related to a standard mobility.

Dissociation of a few protons from a molecule or their addition to a molecule (protonation) does not change the mass or the size of the migrating substances a great deal, whereas in aqueous solution the degree of hydration of an ionic species generally increases with increasing charge.

Consequently the mass M in eqn. 14 should be expressed as a sum of the mass of the molecule itself and the mass of the water of hydration. Moreover, the degree of hydration depends on the intrinsic molecular weight and/or the chemical structure. The degree of hydration decreases with increasing molecular weight[22] and is lower in aromatic than in aliphatic compounds. If a discrepancy between the observed and the calculated mobilities is due to a mass increment as a result of hydration, we can get eqn. 15 using eqn. 14

$$\frac{U_{\text{cal.}}}{U_{\text{obs.}}} = \frac{\sqrt{M + \Delta M}}{\sqrt{M}} \cdot \frac{a + b\sqrt{M}}{a + b\sqrt{M + \Delta M}} \tag{15}$$

where $U_{\text{cal.}}$ is the calculated relative mobility, $U_{\text{obs.}}$ is the observed relative mobility, and ΔM is the mass increment due to hydration. Eqn. 15 can be altered to eqn. 16:

$$\frac{\Delta M}{M} = \left(\frac{aRu}{a + b\sqrt{M}(1 - Ru)}\right)^2 - 1 \tag{16}$$

$$Ru = \frac{U_{\text{cal.}}}{U_{\text{obs.}}} \tag{17}$$

Z in eqn. 14 is a function of the pK(s) and the pH. Now we can derive eqn. 18 combining eqn. 14 with eqn. 13:

$$U_{\text{cal.}} = \frac{1}{2}\left\{\left(\frac{a}{\sqrt{M}} + b\right)\sum_{i=1}^{m+n}\left(1 + \tanh\left[\frac{2.303}{2}(\text{p}K_i - \text{pH})\right]\right)\right\} - n$$

$$U_{\text{cal.}} = -\frac{1}{2}\left\{\left(\frac{a}{\sqrt{M}} + b\right)\sum_{i=1}^{m+n}\left(1 + \tanh\left(\frac{2.303}{2}(\text{pH} - \text{p}K_i)\right)\right)\right\} + m \tag{18}$$

The exact mass M in eqn. 18 should rather be represented as the mass of the hydrated ion as mentioned above.

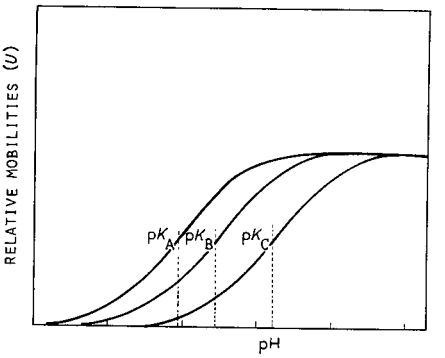

Fig. 5. pH dependence of the electrophoretic mobility. A, B, C: substances with equal molecular weight but different pK values.

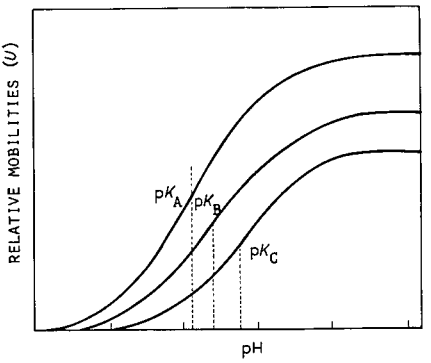

Fig. 6. pH dependence of the electrophoretic mobility. A, B, C: substances with increasing molecular weight and also increasing pK values.

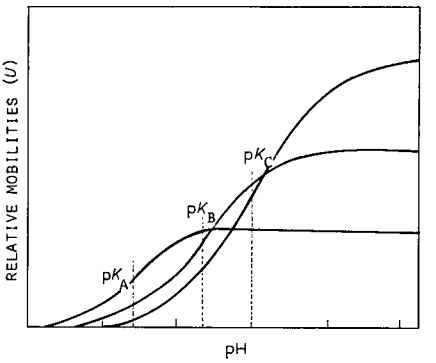

Fig. 7. pH dependence of the electrophoretic mobility. A, B, C: substances with decreasing molecular weight but increasing pK values.

TABLE I

CALCULATED RELATIVE MOBILITIES OF AMINO ACIDS AND THEIR pK VALUES[25,26]

Amino acid	*Mol. wt.*	U_1	pK_1	pK_2	pK_3
Alanine (Ala)	89.1	0.85	2.43	9.69	
β-Alanine (β-Ala)	89.1	0.85	3.60	10.19	
α-Aminobutyric acid (α-Amin)	103.1	0.77	2.55	9.60	
Arginine (Arg)	174.2	0.55	2.17	9.64	12.48
Asparagine (Asn)	132.2	0.66	2.02	8.8	
Aspartic acid (Asp)	133.1	0.66	2.1	3.86	9.82
L-Cysteine (Cys)	121.2	0.70	1.71	8.33	10.78
Cystine (Cysti)	240.3	0.44	<1	2.1	8.71
Glutamic acid (Glu)	147.1	0.62	2.19	4.25	9.67
L-Glutamine (Glu NH_2)	146.2	0.62	2.17	9.13	
Glycine (Gly)	75.1	0.94	2.34	9.60	
Histidine (His)	155.2	0.59	1.78	5.97	8.97
L-Hydroxyproline (Hyp)	131.1	0.66	1.82	9.65	
Isoleucine (Ile)	131.2	0.66	2.26	9.62	
Leucine (Leu)	131.2	0.66	2.36	9.60	
Lysine (Lys)	146.2	0.62	2.20	8.90	10.28
Hydroxylysine	162.2	0.58	2.13	8.62	9.67
Methionine (Met)	149.2	0.61	2.28	9.21	
Ornithine (Orn)	132.2	0.66	1.94	8.65	10.76
Phenylalanine (Phe)	165.2	0.57	1.83	9.13	
Proline (Pro)	115.1	0.72	1.99	10.60	
Serine (Ser)	105.1	0.77	2.21	9.15	
Taurine (Tau)	125.2	0.68	1.5	8.74	
Threonine (Thr)	119.1	0.71	2.15	9.12	
Tryptophan (Try)	204.2	0.49	2.38	9.36	
Tyrosine (Tyr)	181.2	0.53	2.20	9.11	10.07
Valine (Val)	117.2	0.71	2.32	9.62	
$(Gly)_2$	130.2	0.67	3.12	8.17	
Ala–Gly	146.2	0.617	3.11	8.18	
Gly–Tyr	238.3	0.438	2.98	8.40	10.40
Leu–Gly	188.3	0.519	3.18	8.29	
Gly–GluNH_2	203.3	0.491	2.88	8.29	
$(Gly)_3$	186.3	0.522	3.26	7.91	

ESTIMATION OF THE SEPARABILITY OF A MIXTURE

The separability of a mixture A, B, C, ... with different pK(s) and masses is discussed for various cases below.

(1) If the masses of the different ionic species A, B, C, ... are equal and the number of dissociable protons in every species is one:

$$M_A = M_B = M_C = \ldots$$

$$pK_A < pK_B < pK_C > \ldots \quad (19)$$

$$pK_{max} - pK_{min} < 4$$

pK_{max} and pK_{min} are the maximum and the minimum pK values among all the pK values of the substances to be separated. In this case the optimum pH value for the separation is:

$$pH = \frac{pK_A + pK_B + pK_C + \ldots}{n} \quad (20)$$

where n is the number of substances to be separated (see Fig. 5).

(2) If the relation between the masses and the pK values of the substances is:

$$M_A < M_B < M_C < \ldots$$

$$pK_A < pK_B < pK_C < \ldots \quad (21)$$

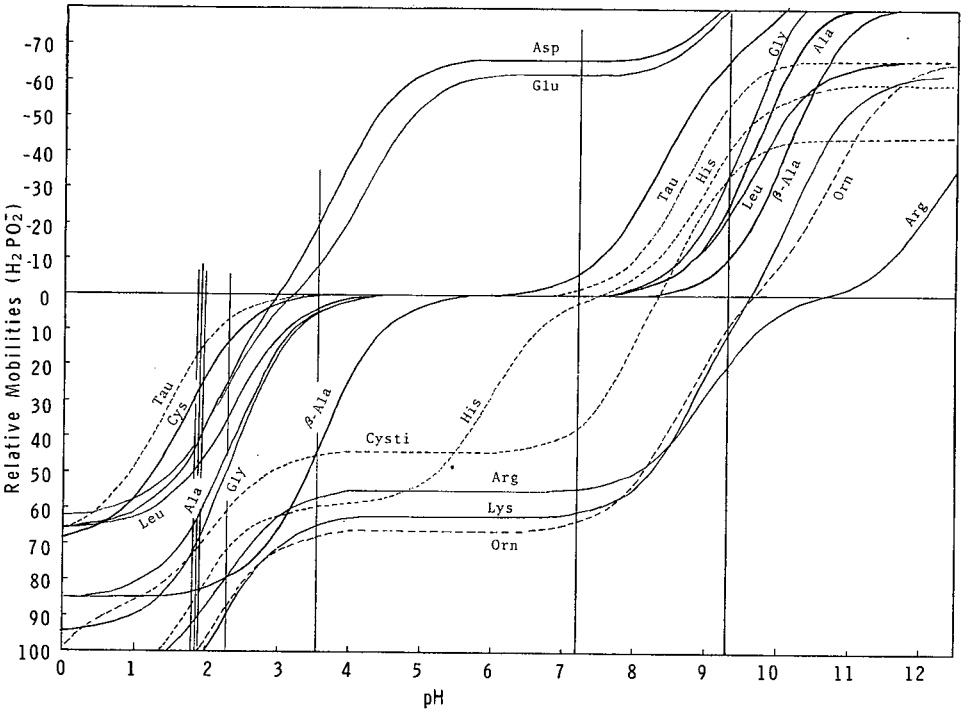

Fig. 8. pH dependence of the electrophoretic mobilities of amino acids.

In this case the separation is easy in the range pH $> pK_{max} - 2$ (see Fig. 6).

(3) If the relation between the masses and pK values is:

$$M_A > M_B > M_C > \ldots$$
$$pK_A < pK_B < pK_C < \ldots \quad (22)$$

In this case the optimum pH for a separation cannot be determined without consulting the zone mobility–pH curves for those substances (see Fig. 7).

CALCULATION OF ZONE MOBILITY–pH CURVES OF AMINO ACIDS

To obtain the zone mobility–pH curves of polyanionic substances the calculation of zone mobilities of monoanionic substances first is essential. Eqn. 23 was derived from experimental data[22]:

$$U_1 = 10 \frac{1}{\sqrt{M}} - 0.21 \quad (23)$$

TABLE II

COMPARISON BETWEEN OBSERVED AND CALCULATED RELATIVE MOBILITIES OF AMINO ACIDS

Compounds	*pH 1.81*					*pH 1.85*				
	Cal.	*Cal.*[c]	*Obs.*[a]	*Obs.*[c]	*Δ %*	*Cal.*	*Cal.*[d]	*Obs.*[b]	*Obs.*[d]	*Δ %*
Ala	0.65	119	100	123	−4	0.64	77	100	69	− 8
β-Ala						0.834	100	145	100	0
α-Amin						0.646	77	90	62	−16
Arg						0.919	110	131	90	−20
Asn						0.393	47	71	49	2
Asp	0.354	65	59	73	8	0.339	41	61	42	1
Cys						0.293	35	60	41	6
Cysti						0.313	38	59	41	3
Glu	0.433	79	66	82	3	0.421	50	67	48	− 2
$GluNH_2$						0.417	50	69	47	− 3
Gly						0.712	85	114	79	− 6
His						0.860	107	131	90	−17
Hyp						0.32	38	54	37	− 1
Ile	0.517	95	80	99	4	0.477	57	77	53	− 4
Leu	0.517	95	78	96	1	0.507	61	77	53	− 8
Lys						1.043	125	147	102	−23
Met						0.442	53	71	49	− 4
Orn						1.012	121	152	105	−16
Phe	0.290	53	61	75	12	0.291	35	61	42	7
Pro	0.435	80	69	85	5	0.417	50	69	48	− 2
Ser	0.547	100	81	100	0	0.533	64	83	57	− 7
Tau						0.212	25	30	20	− 5
Thr	0.484	89	73	90	1	0.470	56	75	52	− 4
Try						0.377	45	46	32	−13
Tyr	0.378	69	53	65	−4	0.368	44	53	37	− 7
Val	0.545	100	81	100	0	0.532	64	81	56	− 1

[a] 2.0% (w/v) formic acid–20% (w/v) acetic acid–0.4 mM cadmium acetate; 100 V/cm, mobility relative to alanine[27].

[b] 2.5% (w/v) formic acid–7.8% (w/v) acetic acid; 100 V/cm; mobility relative to alanine[27].

[c] Standard: valine.

[d] Standard: β-alanine.

TABLE III

COMPARISON BETWEEN OBSERVED AND CALCULATED RELATIVE MOBILITIES OF AMINO ACIDS

Compounds	*pH 1.9*									
	Cal.	*Cal.*[c]	*Obs.*[a]	*Obs.*[c]	*Δ %*	*Cal.*	*Cal.*[c]	*Obs.*[b]	*Obs.*[c]	*Δ %*
Ala	0.622	90	24.2	90	0	0.622	90	100	87	— 3
β-Amin	0.632	92	21.7	82	—10					
Aspn						0.375	55	73	63	— 8
Asp	0.403	59	8.0	70	11					
Cys	0.342	50								
Cysti	0.302	43	14.3	53	10	0.302	50	67	58	— 8
Glu	0.405	59	15.5	58	— 1					
Gly	0.688	100	26.9	100	0	0.688	100	115	100	0
Hyp	0.302	44	130	48	— 4	0.302	44	60	52	8
Leu	0.490	71	20.9	78	6					
Lys						1.03	149	141	127	—22
Met	0.429	62	17.6	67	5					
Orn						1.01	146	141	123	—23
Phe	0.261	38	16.6	63	25	0.261	38	62	54	16
Pro	0.398	58	15.4	56	— 2	0.398	58	75	65	7
Ser	0.513	75	19.7	74	— 1					
Tau	0.106	15	6.4	22	7					
Thr	0.595	87	18.2	67	—10	0.595	87	78	68	—29
Try	0.367	53	15.5	59	6	0.367	53	48	42	—11
Tyr	0.354	52	16.2	59	7					
Val	0.515	75	21.2	78	3					

[a] 2 *N* acetic acid–0.6 *N* formic acid (1:1), pH 1.9; Whatman No. 1 paper; 70 V/cm; 200 min; unit is cm[28].

[b] Acetic acid–formic acid–water (150:50:800, v/v/v), pH 1.9; Whatman No. 3 filter paper; 85 V/cm; cooling fluid temperature, −5°; mobility relative to that of alanine[29].

[c] Standard: glycine.

U_1 was the zone mobility of monoanionic substances relative to that of monoanionic hypophosphorous acid as migration standard. The calculated relative zone mobilities of monoanions of amino acids, which were calculated using eqn. 23 and their pK values, are given in Table I. Fig. 8 shows the U–pH curves of amino acids derived from eqn. 18 and Table I. The relative zone mobilities of di-cations and di-anions generated by proton association and dissociation were just twice as large as those of the monoanions shown in Table I. In Tables II–V, observed and calculated mobilities at various pHs of the background solutions are recorded. The calculated values are obtained with eqn. 18. The comparison of the calculated with the experimental values is simplified giving ratios of the relative zone mobilities and showing them in a clear graph (see Figs. 9 and 10). From these figures it can be seen that the calculated and the experimental values are usually in good agreement. The crossing of a few connecting lines in this graph means that the migration order found experimentally is different from the expected one. Those amino acids deviating rather often from the theory are phenylalanine, tryptophan, tyrosine, lysine, arginine, ornithine, and histidine. Hydrophobic phenyl groups are present in the phenylalanine, tryptophan, and tyrosine molecules. In lysine, ornithine, arginine, and histidine the mean charge in the low pH range is greater than unity whereas in the other amino acids the mean charge in this pH range is usually less than unity. The greater charge gives rise to

TABLE IV

COMPARISON BETWEEN OBSERVED AND CALCULATED RELATIVE MOBILITIES OF AMINO ACIDS

Compounds	*pH 2.3*					*pH 3.3*				
	Cal.	*Cal.*[c]	*Obs.*[a]	*Obs.*	*Δ %*	*Cal.*	*Cal.*[a]	*Obs.*[b]	*Obs.*[a]	*Δ %*
Ala	0.444	125	15	117	— 8	0.084	14	5	7	—7
β-Ala						0.565	93	61	90	—3
α-Amin	0.495	139	13.1	102	—37					
Arg						0.585	96	68	100	4
Asp	—0.153	—43	—6.8	—53	—10	—0.167	—27	—21	—31	4
Cysti	0.166	47	8.5	66	19					
Glu	0.268	76	3.8	30	—46	—0.027	— 4	— 7	—10	7
Gly	0.493	139	16.4	128	—11	0.094	15	7	10	—5
His						0.609	100	68	100	0
Hyp	0.165	47	8.0	63	16					
Leu	0.354	100	12.8	100	0					
Met	0.297	84	10.9	85	1					
Phe	0.143	40	10.1	79	39					
Pro	0.239	68	9.6	75	7	0.033	5	0	0	—5
Ser	0.309	86	11.9	93	7					
Tau	0.116	33	5.8	45	12					
Thr	0.293	83	11.2	88	5					
Tyr	0.236	67	9.3	73	6					
Val	0.348	98	12.8	100	2					

[a] 1 *N* acetic acid–0.6 *N* formic acid (1:1), pH 2.3; Whatman No. 1 paper; 70 V/cm; 180 min[28].
[b] Pyridine–formic acid buffer, pH 3.3; Whatman No. 4 paper; 10 V/cm; 1–2 h; mobility relative to amaranth[30].
[c] Standard: leucine.
[d] Standard: histidine.

TABLE V

COMPARISON BETWEEN OBSERVED AND CALCULATED RELATIVE MOBILITIES OF AMINO ACIDS

Compound	*pH 7.2*					*pH 9.3*				
	Cal.	*Cal.*[c]	*Obs.*[a]	*Obs.*[c]	*Δ %*	*Cal.*	*Cal.*[c]	*Obs.*[b]	*Obs.*[c]	*Δ %*
Ala						—0.25	—28.5	—10	— 12.3	17
β-Ala						—0.098	— 11.2	— 3	— 4	7
Arg	0.55	83	62	81	—2	0.338	39	45	56	17
Asp	—0.66	—100	—76	—100	0	—0.876	—100	—81	—100	0
Glu	—0.61	— 94	—72	— 95	1	—0.824	— 94.0	—77	— 95	1
Gly	0.0	0.0	0	0	0	—0.317	— 36.2	—12	— 15	16
His	0.08	12	8	11	—1	—0.189	— 22	—10	— 12	10
Pro	0.0	0.0	0	0	0	—0.003	— 3	0	0	— 3
Ser						—0.447	— 51	—28	— 35	16

[a] Dimethylaminopropionitrile–acetic acid buffer; Whatman No. 4 paper; 10 V/cm; 1–2 h; mobility relative to amaranth[30].
[b] 2-Dimethylaminoethanol–acetic acid buffer[30].
[c] Standard: aspartic acid.

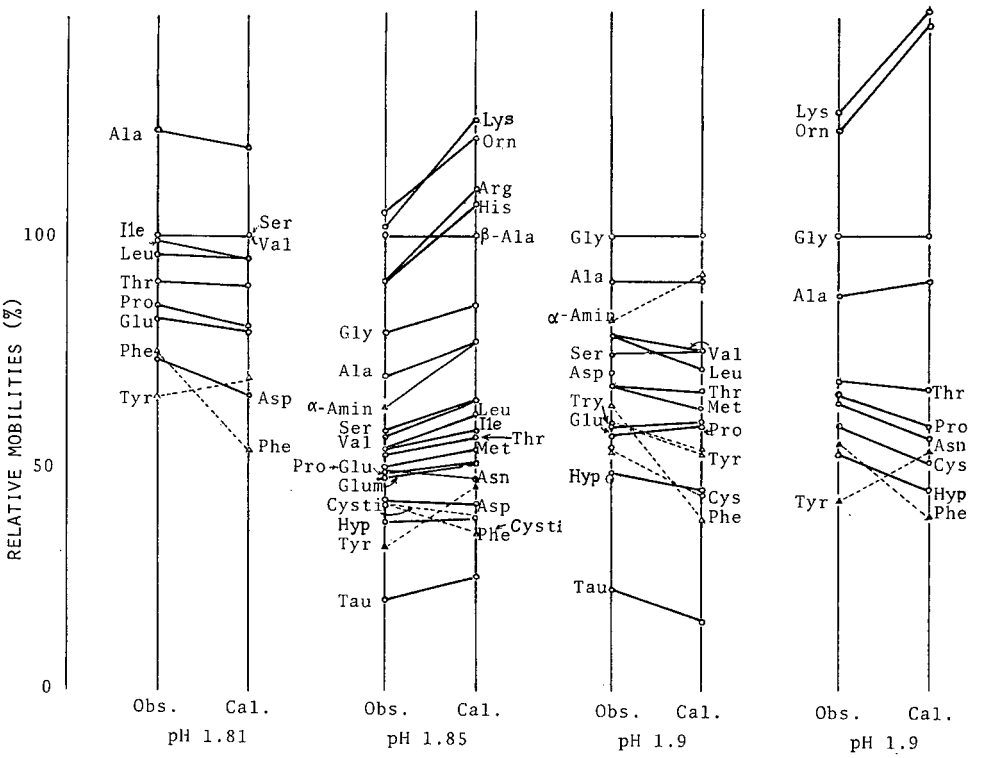

Fig. 9. Schematic comparison between observed and calculated relative mobilities of various amino acids. Observed data published by different authors.

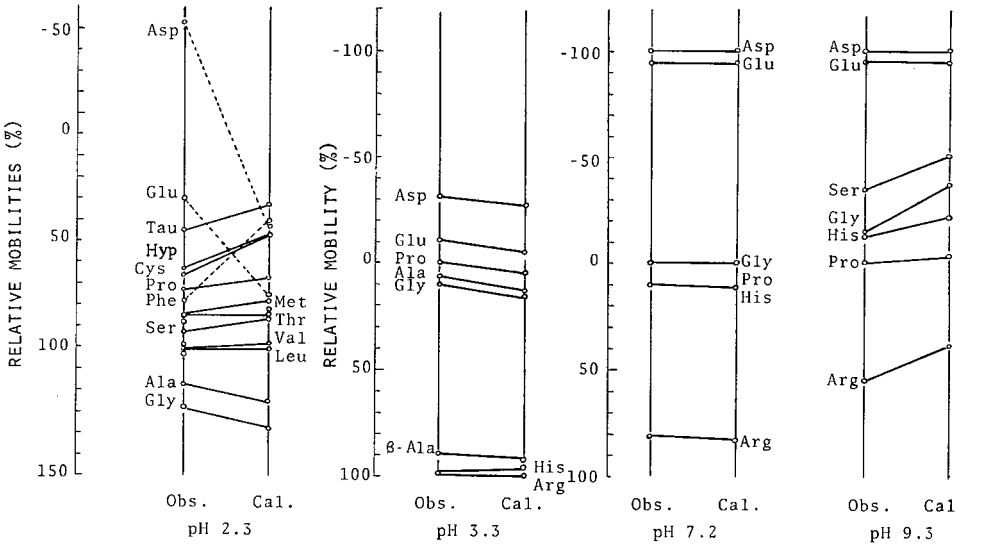

Fig. 10. Schematic comparison between observed and calculated relative mobilities of various amino acids at different pHs.

a higher degree of hydration which is neglected in the calculations hence leading to calculated mobility values which consequently are too high. At pH 7.2 and 9.3 (Table V) the charges of all amino acids are about equal and thus the calculated and the experimental mobilities are in good agreement here.

TABLE VI

COMPARISON BETWEEN THE OBSERVED AND THE CALCULATED RELATIVE MOBILITIES OF SOME PEPTIDES AT pH 1.9

Compound	*Cal.*	*Obs.*[a]	*Cal.*[c]	*Obs.*[a,c]	*Δ %*	*Cal.*[d]	*Obs.*[b,d]	*Δ %*
Gly	0.688	16.4	109.7	68.6	−41.1			
Ala	0.622					100	100	0
$(Gly)_2$	0.627	23.9	100	100	0			
Ala–Gly	0.554	22.8	88.4	95.4	7.0			
Gly–Tyr	0.404	16.5	64.4	69.0	4.6	64.9	64	0.9
Leu–Gly	0.492	19.4	78.5	81.2	2.7	79.1	81	0.9
Gly–GluNH$_2$	0.465					74.7	82	7.3
$(Gly)_3$	0.499	21.4	79.6	89.5	9.9			

[a] Buffer (2 *N* acetic acid–0.6 *N* formic acid, 1:1, v/v); Whatman No. 1 paper; 70 V/cm; 60 min; unit is cm[28].

[b] Buffer (glacial acetic acid–formic acid–water, 150:50:800, v/v/v); Whatman No. 3 paper; 85 V/cm; mobility relative to alanine[29].

[c] Standard: $(Gly)_2$.

[d] Standard: alanine.

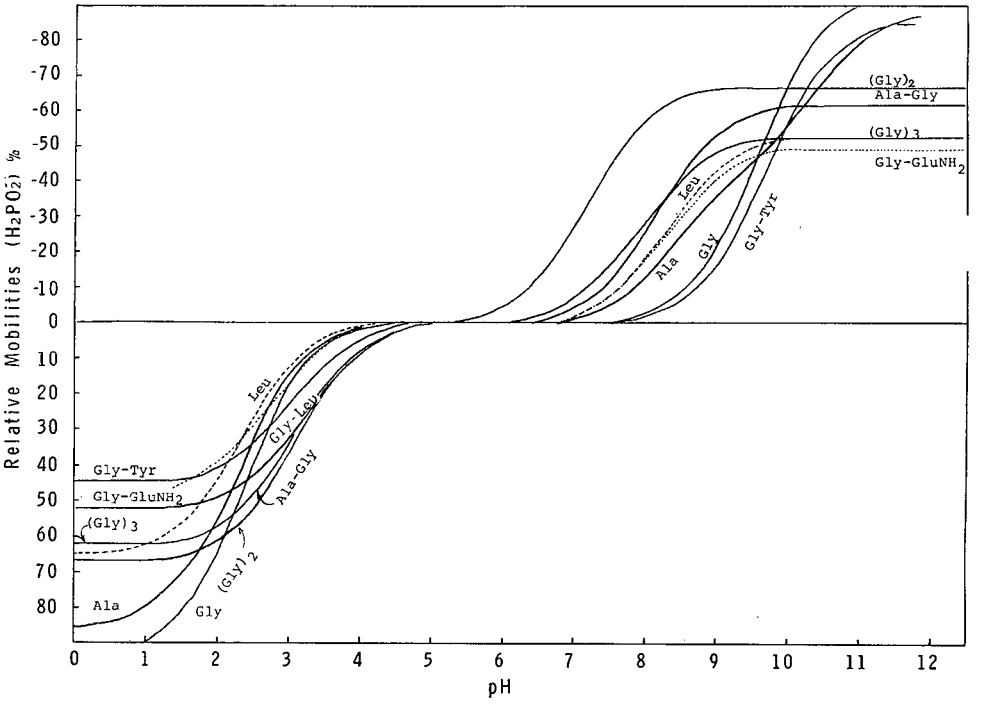

Fig. 11. pH dependence of the relative mobilities of selected oligopeptides.

Table VI shows a comparison between the observed and the calculated mobilities of a few peptides at pH 1.9. The negative percentage deviation of glycine in Table VI is very large. This is due to a high degree of hydration relative to that of the other dimer or trimer peptides. On the other hand, the positive percentage deviation of $(Gly)_3$ shows a lower degree of hydration in comparison with that of the other dimer and trimer amino acids. The observed mobilities of the other oligopeptides agree fairly well with the calculated ones. A few mobility–pH curves of amino acids and oligopeptides are given in Fig. 11. The pH value of the background electrolyte suitable for the separation of a certain mixture can be read easily from this graph. Below pH = 1 and above pH = 11 the mobility depends only on the difference in the weights of the species and not on the pH of the background solution. Between pH 4 and 6 the peptides have no or only a negligibly small charge and hence do not migrate at all. The optimum pH for a separation will vary according to which species are to be separated.

The simplicity and rapidity of this method compared with finding the optimum pH from trial and error tests is obvious in such complicated cases.

REFERENCES

1 J. R. WHITAKER, *Paper Chromatography and Electrophoresis*, Vol. 1, Academic Press, New York and London, 1967.
2 M. BIER (Editor), *Electrophoresis, Theory, Methods and Applications*, Academic Press, New York and London, 1967.
3 Y. KISO, *Kagaku No Ryoiki*, 23 (1969) 45 and 283
4 V. SHVEDOV AND A.V. STEPANOV, *Radiokhimiya*, 1 (1959) 162.
5 Y. KISO, *J. Sci. Hiroshima Univ.*, 27 (1963) 17.
6 V. JOKL, *Cesk. Farm.*, 12 (1963) 44.
7 M. MAZZEI AND M. LEDERER, *J. Chromatogr.*, 31 (1967) 196.
8 M. LEDERER AND M. MAZZEI, *J. Chromatogr.*, 35 (1968) 201.
9 H. PEETERS, *Adv. Clin. Chem.*, 2 (1959) 1.
10 G. G. STOKES, *Trans. Camb. Phil. Soc.*, 9 (1856) 5.
11 J. C. M. LI AND P. CHANG, *J. Chem. Phys.*, 23 (1955) 518.
12 S. GLASSTONE, K. J. LAIDLER AND H. EYRING, *The Theory of Rate Processes*, McGraw Hill, New York, 1941, pp. 519, 552.
13 J. T. EDWARD, *Chem. Ind. (London)*, (1956) 929; *Sci. Proc. Roy. Dublin Soc.*, 27 (1956) 273.
14 V. JOKL, *J. Chromatogr.*, 13 (1964) 451.
15 H. G. KUNKEL AND A. TISELIUS, *J. Gen. Physiol.*, 35 (1951) 89.
16 J. T. EDWARD, *J. Chromatogr.*, 1 (1958) 446.
16a R. CRAWFORD AND J. T. EDWARD, *Anal. Chem.*, 29 (1957) 1543.
17 H. WALDMANN-MEYER, *Chromatogr. Rev.*, 5 (1963) 1.
18 J. T. EDWARD, *Adv. Chromatogr.*, 2 (1966) 62.
19 J. T. EDWARD AND R. CRAWFORD, *J. Chromatogr.*, 1 (1958) 449.
20 J. T. EDWARD AND D. WALDRON-EDWARD, *J. Chromatogr.*, 20 (1965) 563.
21 R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 40 (1946) 33.
22 Y. KISO, M. KOBAYASHI, Y. KITAOKA, K. KAWAMOTO AND J. TAKADA, *J. Chromatogr.*, 36 (1968) 215.
23 W. PREETZ AND E. BLASIUS, *Z. Anorg. Allg. Chem.*, 332 (1964) 140.
24 E. BLASIUS AND W. PREETZ, *Z. Anorg. Allg. Chem.*, 335 (1965) 16.
25 R. M. C. DAWSON, D. C. ELLIOTT, W. H. ELLIOTT AND K. M. JONES (Editors), *Data for Biochemical Research*, Clarendon Press, Oxford, 1959, p. 2.
26 K. NARITA AND T. MURAKAMI (Editors), *Kuromatogurafii no Jissai*, Vol. I, Hirokawa Shoten, Tokyo, 1966, p. 116.
27 G. N. ATFIELD AND C. J. O. R. MORRIS, *Biochem. J.*, 81 (1961) 606.
28 B. KICKHOEFEN AND O. WESTPHAL, *Z. Naturforsch.*, 7B (1952) 659.
29 Z. PRUSIK AND B. KEIL, *Collect. Czech. Chem. Commun.*, 25 (1960) 2049.
30 L. N. WERUM, H. T. GORDON AND W. THORNBURG, *J. Chromatogr.*, 3 (1960) 125.

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THE ISOLATION OF PROMETHIUM BY DISPLACEMENT CHROMATOGRAPHY DUE TO COMPLEX FORMATION*

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SUMMARY

The separation of a mixture of rare earth elements by means of solutions of ethylenediaminetetraacetic, diethylenetriaminepentaacetic and nitrilotriacetic acids is carried out. It is shown that nitrilotriacetic acid has the minimum value for the height equivalent to a theoretical plate. The high separation power of nitrilotriacetic acid is accounted for by the greater velocity of the diffusion processes as compared to the other complexones. The influence of kinetic factors on the height equivalent to a theoretical plate value in the elution of cerium and neodymium by diethylenetriaminepentaacetic acid solution is investigated as an example. The limiting stage for elution from a resin containing no more than 12% divinylbenzene, is shown to be the outer diffusion inhibition.

The method of displacement chromatography by complex formation can be used for separating the rare earth elements (r.e.e.). This method has been used successfully for preparing the stable isotopes of r.e.e., as well as for isolating radioactive r.e.e. in considerable amounts[1,2].

The present communication is devoted to the use of this method for the isolation of promethium-147 from fission products.

A peculiarity concerning the isolation of the radioactive products is the necessity of completing an effective separation procedure over a minimum space of time in order to reduce radiation damage which could cause destruction of the resin and eluent and thus losses in the product being isolated.

In practice, considerable success has been achieved using the above-mentioned method, but theoretical studies relating to the conditions and the determination of the separation efficiency have only been reported recently[3].

The efficiency of a chromatographic separation is determined by kinetic and thermodynamic parameters according to the theoretical conception proposed by POWELL AND SPEDDING[1] and is characterized by eqn. 1

$$\log \frac{c_A}{c_B} = \frac{\log \alpha_B^A}{h} \cdot l \qquad (1)$$

* Presented at the 3rd Russian–Italian Symposium on Chromatography, Tbilisi, October 26th–29th, 1970.

where

c_A and c_B are the concentrations of the ions in solution,

α_B^A is separation factor for vicinal r.e.e.,

h = height equivalent to a theoretical plate (HETP),

l = distance along the axis of the column from the middle of the front.

If one compares the separation factors of promethium from its neighbours, samarium and neodymium, in the presence of those complexones most frequently used for separations, one would give some preference to diethylenetriaminepentaacetic acid (DTPA) and nitrilotriacetic acid (NTA), as opposed to ethylenediaminetetraacetic acid (EDTA) and hydroxyethylethylenediaminetriacetic acid (HEDTA) (Table I).

However, it was found experimentally[2] that NTA is notable for its considerably greater efficiency when carrying out the separation procedure as compared with the rest of the complexones.

This greater efficiency of NTA is in the opinion of WHEELWRIGHT[2] connected with the kinetics of the diffusion processes rather than with the thermodynamic parameters.

To compare the kinetic characteristics for these eluents we determined the HETP values on eluting a mixture of r.e.e. with solutions of NTA, DTPA and EDTA. The separation was carried out on several columns combined in sequence. The resin used was KU-2 with a granule size of 60–90 mesh. The results of the separations are given in Figs. 1, 2 and 3 and Table II.

TABLE I

SEPARATION FACTORS FOR Pm/Sm AND Pm/Nd[2]

R.e.e.	*Eluent*			
	EDTA	*HEDTA*	*DTPA*	*NTA*
Pm–Nd	1.8	1.7	2.0	2.3
Pm–Sm	1.8	1.7	2.0	2.3

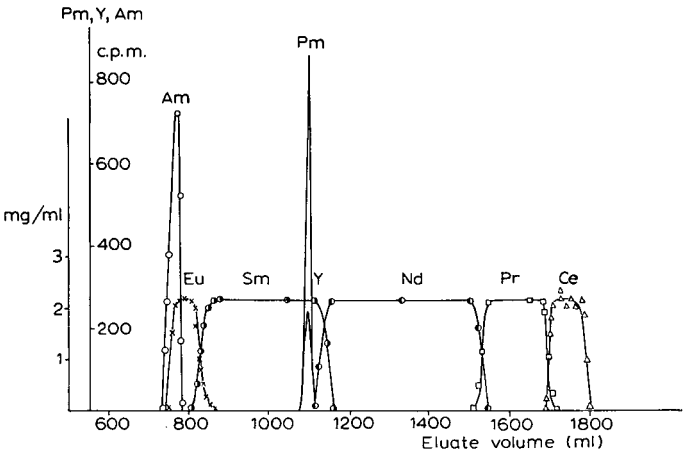

Fig. 1. Separation of the r.e.e. by EDTA solution.

As can be seen from the data in Table II, in order to gain the same degree of separation the least time is spent when NTA is used (the velocity of the band through the column amounts to 16 cm/h); the longest time is when EDTA is used (the velocity of the band is 4.8 cm/h). The band of promethium overlaps with neighbouring bands, since the promethium-147 used for the separation was only present in microquantities.

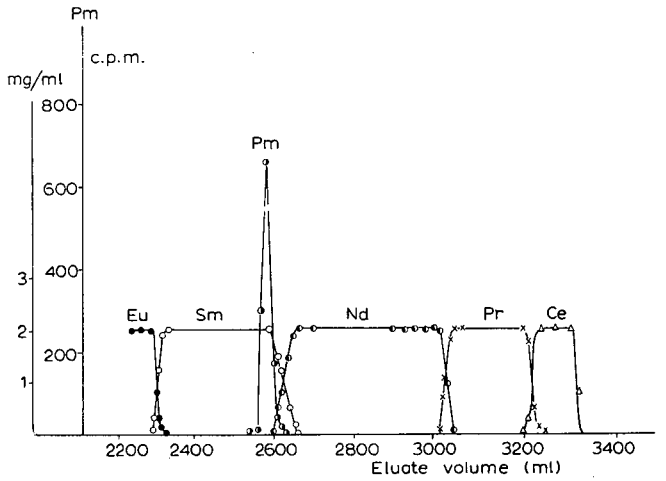

Fig. 2. Separation of the r.e.e. by DTPA solution.

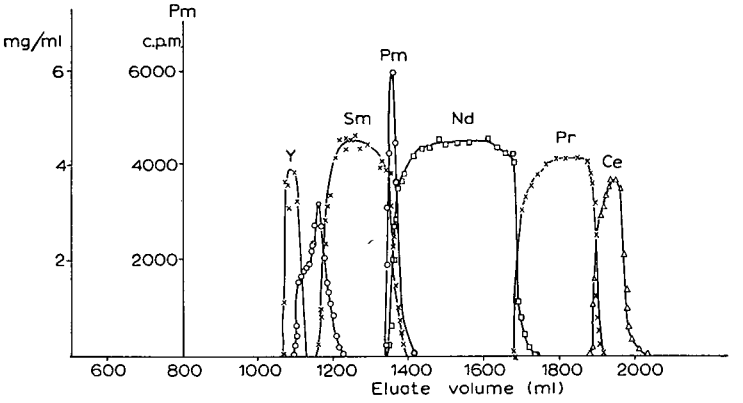

Fig. 3. Separation of the r.e.e. by NTA solution.

The poor elution by means of DTPA (the joint elution of yttrium and promethium) can be avoided by using a mixture of DTPA and EDTA solutions as eluent (Fig. 4).

The determination of the HETP was carried out by using eqn. 1 for the pair praseodymium–neodymium, since the partition factors, under conditions corresponding to those of the separation procedure, have been determined by us for these r.e.e. (Table III).

As would be expected from the data given in Table II, NTA has the least value of HETP when the velocity of the front is greatest. Thus it appears that the greater

TABLE II

COMPARISON OF ELUENT EFFICIENCIES

Eluent	*Columns*	*Experimental conditions*	*Velocity of solution filtration* ($ml/min/cm^2$)	*Velocity of r.e.e. band shift* (cm/h)	*R.e.e. concentration in filtrate* (g/l)	*Quantity of displacement bands,* v	*Separation factor,* α_B^A
0.015 mole/l, pH 8.5, EDTA, $t = 70°$ (Fig. 1)	Sorption column, $H = 86$ cm; $d = 0.86$ cm. Separation columns, $\Sigma H = 450$ cm; $d = 0.38$ cm.	Resin KU-2 60–90 mesh Cu-form	3.7	4.8	1.8	1.6	1.8
0.025 mole/l, pH = 6.0, DTPA, $t = 70°$ (Fig. 2)	Sorption column, $H = 70$ cm; $d = 0.96$ cm. Separation columns, $\Sigma H = 286$ cm; $d = 0.38$ cm.	Resin KU-2 60–90 mesh H-form	3.5	10.00	2.2	1.35	2.2
0.1 mole/l, pH = 7.0, NTA, $t = 70°$ (Fig. 3)	Sorption column, $H = 100$ cm; $d = 0.86$ cm. Separation columns, $H_1 = 93$ cm; $d_1 = 0.80$ cm; $H_2 = 97$ cm; $d_2 = 0.80$ cm; $H_3 = 93$ cm; $d_3 = 0.40$ cm.	Resin KU-2 60–90 mesh Zn-form	4.0	16.0	4.5	1.6	2.2

efficiency of NTA, compared to EDTA and DPTA, is perhaps connected with the greater velocity of the diffusion processes.

To find the optimum conditions for separating r.e.e. by means of different complexones, we then considered the influence of kinetic parameters on the HETP value. The data obtained allow one to ascertain the mechanism limiting the process in its stationary state in the case of a convex sorption isotherm and to find the most favourable conditions for separation.

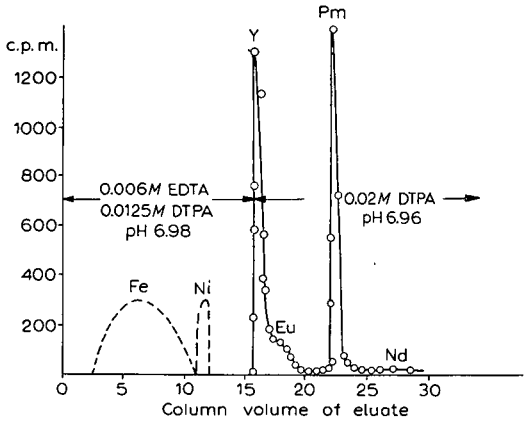

Fig. 4. Separation of the r.e.e. by a mixture of eluents. The elution is first performed with a mixture of 0.006 mole/l EDTA and 0.025 mole/l DTPA solutions at pH 6.98. It is then continued with 0.02 mole/l DTPA solution at pH 6.96. Temperature 70°; the sorption column was 60 cm long by 0.86 cm wide. Separation column: $H = 95$ cm, $d = 0.37$ cm. Flow rate: 3.5 ml/min/cm^2. The number of substitution bands was 0.30. The rate of zone displacement was 5 cm/h.

TABLE III

PARTITION FACTORS OF Pr–Nd IN THE PRESENCE OF COMPLEXONES

Complexone	*t* (°C)	α_B^A	*HETP* (*cm*)
EDTA	25	2.12	1.77
	70	1.80	
DTPA	25	3.08	2.30
	70	2.51	
NTA	25	1.78	1.17
	70	2.20	

The quantitative characteristics of eluting the r.e.e. bands were deduced from elution curves for the HETP values according to eqn. 1. With a view to determining the limiting diffusion mechanism a computation of the HETP according to GLUECKAUF's equations[4] (eqns. 2, 3, 4 and 5), reduced to a form convenient for such a calculation[5], was performed.

In the case of film kinetics:

$$\Delta F = \frac{Vc_2 - Vc_1}{0.5q\left(\frac{\overline{X}}{C} + \beta\right)\left(\frac{\alpha_B^A}{\alpha_B^A - 1}\ln\frac{c_2}{c_1} - \frac{1}{\alpha_B^A - 1}\ln\frac{C - c_2}{C - c_1}\right)}, \qquad (2)$$

$$\Delta F = \frac{2\delta r \overline{F}}{(\overline{X} + \beta C) 3D}, \tag{3}$$

where

ΔF is the HETP for outer diffusion,

Vc_1 and Vc_2 are the elution volumes, at which the developing solution has concentrations c_1 and c_2,

$\overline{X}$ is volume capacity of exchanger,

C = equivalent total ion concentration in solution,

δ = the film thickness,

q = cross-section of the column,

r = radius of the particles of the exchanger,

$\overline{F}$ = flow rate,

D = factor of outer diffusion.

In the case of gel diffusion:

$$\Delta G = \frac{Vc_2 - Vc_1}{0.5q\left(\frac{\overline{X}}{C} + \beta\right)\left(\frac{1}{\alpha_B^A - 1} \ln \frac{c_2}{c_1} - \frac{\alpha_B^A}{\alpha_B^A - 1} \ln \frac{C - c_2}{C - c_1}\right)} \tag{4}$$

$$\Delta G = \frac{r^2 \overline{F}}{(\overline{X}/C + \beta) 14 \overline{D}} \tag{5}$$

where

ΔG is the HETP for inner diffusion,

$\overline{D}$ = the factor of inner diffusion.

The experimental and calculated data for the elution of an equimolar mixture of neodymium and cerium by DTPA solution are given in Tables IV–VII.

It can be seen from the experimental data, concerning the influence of velocity and temperature on HETP, that the values of HETP decrease with increasing temperature and increase when the flow rate of the solution is increased.

The dependence of HETP upon the grain size of the resin is linear which is evidence in favour of a film type mechanism. The values of HETP do not change with an increase in the eluent concentration and in the crosslinking from 2 % to 8 % divinylbenzene, which also confirms the theory of a film type mechanism of diffusion (eqn. 3). The value of the HETP only increases up to a degree of cross-linking of 12 %; this would seem to be connected with the passage of the system to a mixed mechanism of diffusion. Such conclusions are in accordance with the results published by HAGIWARA[3].

The experimental results coincide with the conclusions which can be derived from the calculated data. The HETP values calculated from eqn. 1 are near to those calculated according to eqn. 2 for outer diffusion inhibition (columns 4 and 6 of Table VI). The exception is an experiment in which a resin was used which contained 12 % divinylbenzene. The process in this case is determined by a mixed mechanism.

Thus on the grounds of calculated as well as experimental data one can conclude

TABLE IV

INFLUENCE OF TEMPERATURE AND FLOW RATE OF ELUENT ON HETP

DTPA concentration 0.025 mole/l; pH 7.

Test No.	*t* (°C)	*Flow rate* (*ml/min/cm²*)	*HETP* (*cm*)		
			h (*eqn. 1*)	*ΔG*	*ΔF*
1	25	1.87	1.70	2.91	1.62
2	25	1.92	1.84	2.96	1.75
3	25	6.45	2.40	3.41	2.34
4	25	11.32	3.56	6.60	3.45
5	50	2.0	0.96	1.64	1.07
6	50	6.01	1.57	2.81	1.69
7	50	6.43	1.63	3.18	1.87
8	50	6.70	1.61	3.06	1.85
9	50	12.60	2.13	3.54	2.29
10	70	1.63	0.52	0.76	0.49
11	70	2.08	0.64	0.92	0.71
12	70	3.77	0.98	1.23	1.05
13	70	9.02	1.28	1.82	1.22
14	70	9.65	1.34	2.10	1.32
15	70	11.20	1.68	3.16	1.63
16	70	17.62	2.15	4.02	2.30
17	90	2.08	0.42	0.77	0.60
18	90	6.44	1.01	1.81	0.92
19	90	7.06	1.07	2.24	0.96
20	90	14.15	1.62	3.61	1.50

TABLE V

INFLUENCE OF THE GRAIN SIZE OF KU-2X8 ON HETP

DTPA concentration 0.025 mole/l, ionic strength 0.079; flow rate 3.8 ml/min/cm²; temperature 70°.

Test No.	$10^2 r$ (*cm*)	*HETP* (*cm*)			$10^3\delta$ (*cm*)
		h (*eqn. 1*)	*ΔG*	*ΔF*	
21	0.58 ± 0.06	0.78	1.12	0.74	1.26
22	0.72 ± 0.10	0.81	1.04	0.82	1.27
23	1.40 ± 0.29	1.52	2.58	1.46	1.28
24	2.18 ± 0.43	2.29	3.49	2.12	1.27

TABLE VI

INFLUENCE OF DEGREE OF CROSS-LINKING ON HETP

Resin Dowex-50, 100–200 mesh; DTPA concentration 0.025 mole/l, pH = 7, ionic strength 0.079; flow rate 3.8 ml/min/cm²; temperature 70°.

Test No.	$E = \overline{X}\frac{mg-eq^a}{ml}$	% *DVB*	*HETP* (*cm*)		
			h (*eqn. 1*)	*ΔG*	*ΔF*
25	1.33	2	0.93	1.28	0.96
26	1.40	4	0.92	1.23	1.06
27	1.55	8	0.99	1.70	0.99
28	2.18	12	2.40	1.58	1.09

[a] Volume capacity of ionite.

that the outer diffusion inhibition under the conditions studied is the limiting factor for eluting the r.e.e. by a DTPA solution from a resin containing no more than 8% divinylbenzene.

The results obtained allow one to predict, by use of eqn. 1, the zones of neighbouring elements overlapping promethium (observing the condition that HETP values for neighbouring r.e.e. differ very little from one another) and to choose the optimum conditions for separating this element.

TABLE VII

INFLUENCE OF DTPA CONCENTRATION ON HETP

Resin KU-2, 60–90 mesh; ionic strength 0.079; flow rate 4.5 ml/min/cm²; temperature 70°.

Test No.	C_{DTPA} (*mole/l*) $pH = 7$	*HETP* (*cm*)		
		h (*eqn. 1*)	ΔG	ΔF
29	0.01	1.46	4.27	1.47
30	0.025	1.44	2.11	1.39
31	0.05	1.36	3.17	1.48
32	0.050	1.38	3.24	1.59

REFERENCES

1 I. E. POWELL AND F. H. SPEDDING, *Chem. Eng. Prog., Symp. Ser.*, 55 (1959) 101.
2 E. I. WHEELWRIGHT, *J. Inorg. Nucl. Chem.*, 31 (1969) 3287.
3 Z. HAGIWARA, *J. Phys. Chem.*, 73 (1969) 3102.
4 E. GLUECKAUF, *Ion Exchange and its Applications*, London, 1955, p. 34.
5 T. D. SEMENOWSKAJ, *Thesis*, Institute of Physical Chemistry, the U.S.S.R. Academy of Sciences, Moscow, 1966.

Notes

CHROM. 5381

Reproducibility of retention data on porous polystyrene polymers (Porapaks)

A new type of adsorbent — an organic porous polymer based on polystyrene — was developed for gas chromatography (GC) by HOLLIS[1–3]. These materials, manufactured under various commercial names such as Porapak, Chromosorb 101–105, Synachrom, Polypak, etc., have excellent separation properties, especially for compounds of polar character, and their application has therefore been fairly widespread. They have also recently been employed in thin-layer[4] and liquid[5] chromatography.

Numerous papers exist in the literature, in which retention data[2,3,6–9] are tabulated in the form of specific retention volumes, relative retention volumes, and Kováts' indexes. In addition the manufacturers of these materials give many retention data[10,11] in their leaflets. The measurement of physical characteristics based on retention data[12] has also been carried out on polymer materials of Porapak type.

It is known that reproducible batch preparation of polymers and adsorbents is not an easy matter generally. Individual papers dealing with separations on Porapak express, in some cases, different opinions concerning the use of this material for the separation of certain compounds, *e.g.* the separation[11,13] of a mixture of nitrogen, oxygen, carbon monoxide and argon. GOUGH AND SIMPSON[14] have recently shown that the retention behaviour of compounds on Porapak may differ in certain cases depending on the polymer conditioning. Their data are, however, presented for temperatures higher than those recommended by the manufacturers in many cases, and the differences may thus be caused by changes in the Porapak resulting from the high temperature used.

As a result of this, we have compared tabulated retention data published by different authors and carried out measurements of retention data and separation factors of model mixtures on different batches of Porapak Q and on Porapak Q conditioned in various ways, by means of gas and liquid chromatography.

Experimental

The surface areas of individual Porapak samples were measured by the dynamic desorption method[15] in an apparatus for the determination of the specific surface area of adsorbents[16]. Weights of 7–9 mg were used for the surface area measurements.

The analysis of a model mixture by GC was carried out in a Becker Delft Multigraph 409 gas chromatograph. The column used was 80 cm long and had an I.D. of 0.3 cm. The column temperature was 32°, the carrier gas flow rate was 30 ml of H_2 per min. A thermal conductivity detector was used for detection of the separated substances. A mixture of ethylene, propylene and water vapour was used as the model mixture. The compounds were sampled in amounts of about 5×10^{-7} mole. In all cases, the column packing was prepared in such a way that the Porapak Q was con-

ditioned in the column for 15 h in a flow of nitrogen at the appropriate temperature, and then the column was emptied and repacked with the conditioned polymer.

The analysis by liquid chromatography was carried out in an apparatus of own design. Degassed *n*-hexane was used as the mobile phase. A damping system, described by LOCKE[17], was employed to balance pressure pulses generated by the MC 300 piston pump (Mikrotechna N.E., Prague, Czechoslovakia). Porapak samples were filled into straight stainless steel columns, 50 cm long and 0.2 cm I.D. A capacity detector[18] was used for the detection. In order to decrease the broadening of the chromatographic peaks[19], the capillary connecting the column and the detector was shaped. The measurements were carried out at room temperature. Dead volume of the column was measured by the injection of *n*-octane. 2-μl samples of a mixture of dibutyl phthalate (DBP), benzyl alcohol, and benzene, were used for the measurements of the retention times; the flow rate of the mobile phase was 0.33 ml/min. Thermal processing of the Porapak samples was the same as for the gas chromatographic measurements. The conditioned sample was washed with mobile phase overnight at room temperature.

Results

The relative retention volumes of the compounds measured by various authors on different types of Porapak are shown in Table I. All the retention volumes are

TABLE I

RELATIVE RETENTION VOLUMES OF MODEL GASES ON DIFFERENT TYPES OF PORAPAK

Propene = 1.00.

Compound	*Porapak type*	*Relative retention volumes*						
		32°	*26°*		*30°*		*32°*	
		ref. 3	*ref. 2*	*Δ%*	*ref. 10*	*Δ%*	*ref. 11*	*Δ%*
H_2O	P	0.982	0.961	− 2	0.976	− 1	0.942	− 4
	Q	0.364	0.356	− 2	0.369	+ 1	0.331	− 8
	R	2.67	2.67	0	2.67	0	1.77	−34
	S	1.28	1.27	− 1	1.28	0	1.37	+ 7
	T	4.05	3.94	− 3	4.07	+ 1	3.31	−28
C_2H_4	P	0.257	0.286	+11	0.254	− 1	0.324	+26
	Q	0.141	0.142	+ 1	0.144	+ 2	0.179	+27
	R	0.167	0.159	− 5	0.171	+ 2	0.203	+22
	S	0.154	0.168	+ 9	0.154	0	0.188	+22
	T	0.143	0.150	+ 5	0.141	− 1	0.182	+27
C_3H_8	P	1.59	1.65	+ 4	1.58	− 1	1.00	−27
	Q	1.30	1.30	0	1.31	+ 1	1.12	−14
	R	1.21	1.21	0	1.22	+ 1	1.13	− 7
	S	1.11	1.12	+ 1	1.11	0	1.11	0
	T	0.870	0.860	− 1	0.890	+ 2	1.00	+15
CH_3Cl	P	1.73	1.96	+13	1.72	− 1	1.51	−13
	Q	1.17	1.17	0	1.19	+ 2	0.890	−24
	R	—	—	—	—	—	1.29	—
	S	1.32	1.32	0	1.31	− 1	1.30	− 2
	T	1.80	—	—	1.85	+ 3	1.64	− 9
C_2H_2	P	0.293	0.327	+12	0.292	0	0.381	+30
	Q	0.141	0.142	+ 1	0.144	+ 2	0.148	+ 5
	R	0.253	0.262	+ 4	0.257	+ 2	0.262	+ 4
	S	0.185	0.211	+14	0.192	+ 4	0.223	+21
	T	0.353	0.394	+12	0.407	+15	0.407	+15

relative to propene. The relative retention volumes can differ by as much as 20 % or more without considering the type of Porapak. Small differences (up to 6°) in the temperature of the measurement[2,3,10,11] are, however, not large enough to explain the differences in the data tabulated, especially as the greatest differences have been found at the same operating temperature *cf.* refs. 3 and 11.

Relative retention volumes of some other compounds, in this case by entirely different authors[7,9,10], are compared in Table II. The retention data are given relative to ethyl alcohol. The deviations are large and are to 77 % in the case of propionic acid and ethylene glycol. These differences in retention volumes are due to different authors and thus obviously also to the different samples of Porapak Q being used. The same as has been said above holds for the influence of the difference in temperature, which is 6 or 7°.

Relative retention volumes of ethene and water relative to propene and peak resolution R for the pair propene–water measured by the authors for different batches of Porapak Q and for different conditioning temperatures are presented in Table III.

TABLE II

RELATIVE RETENTION VOLUMES ON PORAPAK Q

Ethanol = 1.00.

Compound	*Relative retention volume*				
	157°	*150°*		*163°*	
	ref. 10	*ref. 9*	*Δ%*	*ref. 7*	*Δ%*
Methanol	0.500	0.375	−25	0.615	+23
Isopropanol	1.73	2.00	+16	1.38	−20
n-Propanol	2.27	2.81	+24	2.15	− 5
tert.-Butanol	2.77	3.25	+17	—	—
Ethylether	2.23	2.50	+12	—	—
Formic acid	0.932	—	—	1.38	+48
Acetic acid	1.96	—	—	2.92	+49
Propionic acid	4.77	—	—	8.46	+77
Ethylene glycol	4.77	—	—	8.46	+77
Propylene glycol	8.41	—	—	13.85	+65
Benzene	6.59	—	—	5.38	−18
Hexane	6.14	—	—	9.23	+50
Cyclohexane	7.73	—	—	10.77	+39
Acetone	1.59	—	—	2.15	+35

TABLE III

RELATIVE RETENTION VOLUMES AND PEAK RESOLUTION OF MODEL GASES ON PORAPAK Q OF DIFFERENT PRODUCTION BATCHES AND CONDITIONING PROCEDURES MEASURED BY GAS CHROMATOGRAPHY

Batch No.	*Conditioning temperature (°C)*	*Specific surface area (m^2/g)*	*Relative retention volumes*		*Peak resolution R propene/water*
			Ethylene	*Water*	
516	25	530	0.129	0.725	1.80
516	190	648	0.130	0.730	1.70
555	25	592	0.123	0.610	2.46
555	190	621	0.125	0.516	3.46
631	25	550	0.128	0.535	2.98
631	190	655	0.130	0.525	3.08

When the Porapak was conditioned at 190° the relative retention volume of water was 0.516 for Porapak batch No. 555; 0.525 for batch No. 631 and even 0.730 for batch No. 516. The peak resolution decreases in the opposite direction. The influence of different conditioning on the relative retention volume and peak resolution varies according to different batches. While being very small with the batches Nos. 516 and 631 (the differences seem to be caused by the error of the measurements), this effect is quite obvious with batch No. 555. The change in peak resolution is also quite considerable. These changes are illustrated in Fig. 1.

The specific surface areas differ to a certain extent, but any evidence of a relationship between the changes in the retention behaviour of various substances and the specific surface area of the Porapak does not seem to exist (Table III).

TABLE IV

RELATIVE RETENTION VOLUMES AND PEAK RESOLUTION OF MODEL SUBSTANCES ON PORAPAK Q OF DIFFERENT PRODUCTION BATCHES AND CONDITIONING PROCEDURES MEASURED BY LIQUID CHROMATOGRAPHY

Batch No.	*Conditioning temperature (°C)*	*Relative retention volume*		*Peak resolution R*	
		DBP	*Benzyl alcohol*	*DBP/benzene*	*Benzyl alcohol/DBP*
516	25	4.07	14.0	1.10	1.67
516	190	4.10	14.2	1.21	1.74
631	190	2.36	8.95	0.56	1.36

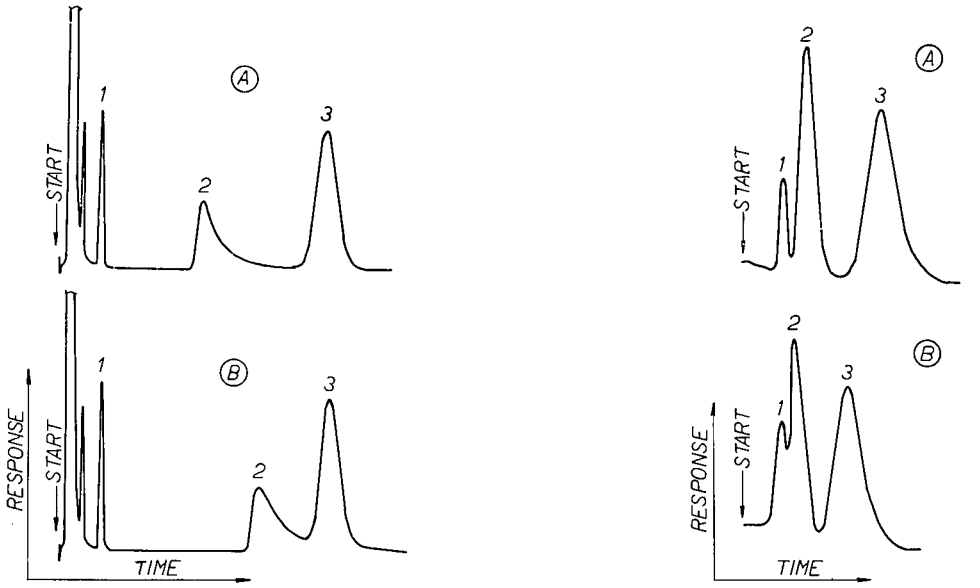

Fig. 1. Gas chromatogram of a model mixture on Porapak Q conditioned at 190°. 1 = Ethylene; 2 = water; 3 = propene. A = Batch No. 631; B = batch No. 516.

Fig. 2. Liquid chromatogram of model mixture on Porapak Q conditioned at 190°. 1 = Benzene; 2 = DBP, 3 = benzyl alcohol. A = batch No. 516; B = batch No. 631.

The results of the measurements carried out by liquid chromatography are summarised in Table IV. Relative retention volumes of DBP and benzyl alcohol, related to the retention volume of benzene, which was almost constant for all the samples, do not change substantially with different conditioning of the same polymer batch. However, the relative retention volume of DBP decreased by 42 % and that of benzyl alcohol by 37 % on using the polymer from another production batch. Similar changes could also be found in peak resolution. While different ways of conditioning change the R value by 5–10 %, the differences are substantially larger when polymer of another production batch is used. The chromatograms of the mixture mentioned are shown in Fig. 2.

Retention volumes of polar compounds (DPB, benzyl alcohol) on batch No. 631 obviously decrease when compared with the retention volumes of the same compounds on batch No. 516. The same effect may be found for the elution of water on the same materials (Fig. 1) in GC.

Conclusions

From the data presented for retention volumes and peak resolution of model compounds on Porapak Q it is evident that they differ substantially depending on the individual batch used and in some cases also on the method of conditioning both in gas and liquid chromatography. The differences in retention data are so large that the tabulated retention data taken from the literature or those measured on a certain batch of Porapak or as subject to certain conditioning cannot be used practically for the qualitative identification of compounds on other batches. Any measurements of physical characteristics on the basis of retention data are also evidently only valid for the given material measured and have only an instructive significance for the general comparison of various types of Porapak.

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1 O. L. HOLLIS, *Anal. Chem.*, 38 (1966) 309.
2 O. L. HOLLIS AND W. V. HAYES, in A. B. LITTLEWOOD (Editor), *Gas Chromatography 1966*, Inst. Petrol., London, 1967, p. 57.
3 O. L. HOLLIS AND W. V. HAYES, *J. Gas Chromatogr.*, 4 (1966) 235.
4 J. JANÁK, *Chem. Ind.*, (1964) 1137.
5 J. JANÁK, Z. JAGARIĆ AND M. DRESSLER, *J. Chromatogr.*, 53 (1970) 525.
6 S. B. DAVE, *J. Chromatogr. Sci.*, 7 (1969) 389.
7 J. D. BURGER, *J. Gas Chromatogr.*, 6 (1968) 177.
8 J. R. L. SMITH AND D. J. WADDINGTON, *Anal. Chem.*, 40 (1968) 522.
9 J. R. LINDSAY SMITH AND D. J. WADDINGTON, *J. Chromatogr.*, 36 (1968) 145.
10 *Porapak*, Waters Associates, Inc., leaflets, 1965.
11 *Chromatography Packings, Components, Instruments and Services*, Waters Associates, Inc., leaflets, 1970.
12 F. M. ZADO AND J. FABECIĆ, *J. Chromatogr.*, 51 (1970) 37.
13 R. VESPALEC, J. MORÁVEK AND I. PIETRIK, *Elaboration of the First Foundations for the Control of Carbon Dioxide Circuits*, Research Report No. 00 06 04 01, Power Research Institute, Prague, Laboratory Jaslovské Bohunice, 1968.

14 T. A. GOUGH AND C. F. SIMPSON, *J. Chromatogr.*, 51 (1970) 129.
15 F. M. NELSEN AND F. T. EGGERTSEN, *Anal. Chem.*, 30 (1958) 1387.
16 D. KOUŘILOVÁ AND M. KREJČÍ, *Chem. Listy*, in press.
17 D. C. LOCKE, *J. Gas Chromatogr.*, 5 (1967) 202.
18 R. VESPALEC AND K. HÁNA, *J. Chromatogr.*, in press.
19 I. HALÁSZ, H. O. GERLACH, A. KRONENSEIN AND P. WALKING, *Z. Anal. Chem.*, 234 (1968) 98.

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Response of the alkali flame ionisation detector to silicon, tin and lead compounds

The alkali flame ionisation detector (AFID) gives a selective response to compounds, the molecule of which contains certain elements. The response of the AFID is known to be selective for nitrogen[1,2], phosphorus[3,4], and arsenic[2,5]. A selective response has further been found to halogens[3,4], and to sulphur[6]. The selectivity of the AFID response to individual elements differs considerably and depends on the construction of the detector, the alkali metal salt used and the value of the background current (hydrogen flow rate), with the exception of phosphorus in which case the response is always greater than that of the flame ionisation detector (FID)[7–10]. The polarity of the response to halogen and sulphur compounds can be both positive and negative, depending on the above mentioned parameters.

The response of the AFID to group IV elements has not been investigated up to the present time. The response of a single-flame detector to hydrocarbons is known to be lower than that of the FID, while in the case of a double-flame detector no response is obtained at low concentrations of hydrocarbons. A negative response to tin compounds, reproducible only with difficulty, has also been mentioned for the double-flame AFID[5]. An anomalous response to silicon compounds has also been found with the FID[11,12].

The response of the AFID to group IV compounds, silicon, tin and lead, is studied in detail in this paper.

Experimental

An alkali ionisation detector with a jet tip of a pressed alkali metal salt, the construction of which has been described by the authors earlier[6], was employed. This detector was built into a Chrom 3 gas chromatograph (Laboratory Equipment, N.E., Prague). A stainless steel column, 68 cm length and 0.6 cm I.D., filled with 20% polyethylene glycol 1500 coated on Chromosorb W (80–100 mesh) was used for the chromatographic analyses of model compounds. Triethoxymethylsilane, tetraethyltin and tetraethyllead were used as model compounds. The chromatographed compounds were dissolved in hexane and sample of about 5×10^{-7} g were dispensed with a Hamilton microsyringe. The column temperature was 55° in the case of the silicon and tin compounds, 85° in that of the lead compound. The carrier gas flow rate was 60 ml of N_2/min, air flow rate 660 ml/min, hydrogen flow rate was varied in such a way that the required value of the background current was attained.

Results and discussion

The dependence of the AFID response (expressed as relative to the FID response) on the background current for the model compounds of tin, lead and silicon with the use of sodium sulphate is demonstrated in Fig. 1. The response of the AFID to silicon compounds, up to 1×10^{-9} A background current, is lower than that of the FID; above this value of background current it is higher. The response increases with the increasing background current over the whole range of the background current under study. The lower response below 1×10^{-9} A is due to the fact that in this range

of background current the detector operates as an FID and since hydrogen flow rate is not optimal, the detector response is not optimal either. This phenomenon of variations and/or additions of the processes in the detector has been described earlier[8].

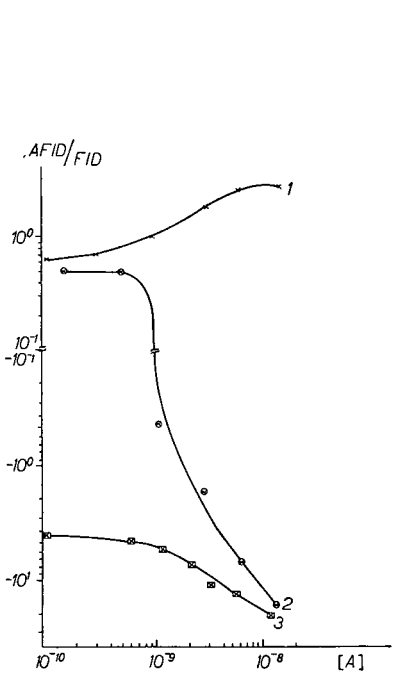
The response of the detector to the lead compound is positive up to approximately 1×10^{-9} A but it is always lower than that of the FID. The reason for the lower response is the same as with the silicon compounds. The response is negative above 1×10^{-9} A. The negative response increases rapidly with increasing background current.

The response of the AFID to tin compounds, over the whole range of the background current, is negative and increases with increasing background current.

The dependence of the response on the background current for a potassium salt is demonstrated in Fig. 2. The response to the silicon compound over the whole range, except for 1.2×10^{-10} A, of the studied background current is higher than that of the FID. The response again increases with the increasing value of the background current.

The response to the lead compound is, with the exception of 1×10^{-10} A, negative over the whole range of the background current examined and its value, similar to that of the tin compound whose response is always negative, again increases with the increasing background current.

In the case of rubidium and cesium salts the dependence of the response to the model compounds on the background current is similar; only the response increases and is highest for cesium.



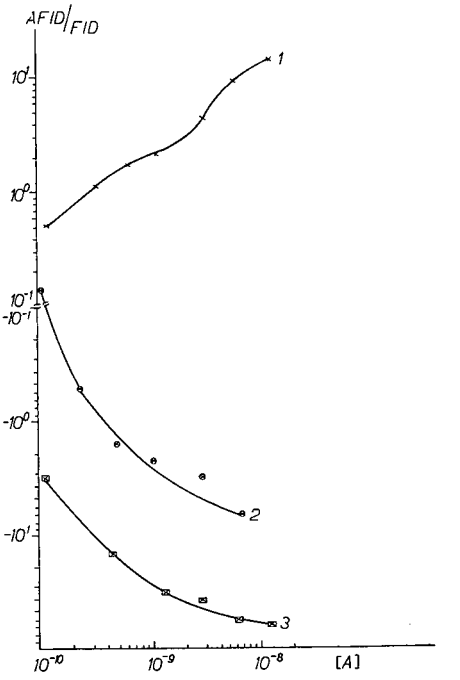


Fig. 1. Dependence of the response on the background current using a sodium salt. 1 = Triethoxysilane; 2 = tetraethyllead; 3 = tetraethyltin.

Fig. 2. Dependence of the response on the background current using a potassium salt. 1 = Triethoxysilane; 2 = tetraethyllead; 3 = tetraethyltin.

The response of the AFID to silicon compounds is thus selective for all the alkali metals in the sense of the response increase, when compared with that of the FID, as in the case of the AFID response to phosphorus and nitrogen compounds[7]. The AFID response to tin and lead compounds is selective not only in the sense of the response increase in contrast to the FID but also in polarity. The value of the background current at which the change in response polarity occurs depends on the type of the alkali metal employed and is, similar[7,8] to the case with sulphur and halogen compounds, the lower, the higher the atomic number of the alkali metal.

In order to compare the molar responses of the AFID used, the ionisation efficiencies at 6×10^{-9} A of all the elements studied are given in Table I. The response is obviously the highest in the case of the sodium salt for phosphorus compounds and decreases for the compounds of the individual elements in the following sequence P > Si > Br > Cl > I. The response to all these compounds is positive. A negative response is only found with this alkali metal to lead and tin compounds. With the use of sodium and background current 6×10^{-9} A the response of the detector to nitrogen and sulphur compounds is not selective and its value is somewhat lower than that of the FID. The highest response with potassium salt is again to phosphorus compounds and decreases further in the following sequence of elements P > Si > N > I. Negative response increases in the sequence of the compounds of Pb < S < Sn. The response of the detector to chloro- and bromo-compounds is, at this value of the background current, nonselective and again somewhat lower than that of the FID. However, it becomes selective at higher values of the background current when it is negative.

TABLE I

IONISATION EFFICIENCY OF THE COMPOUNDS CONTAINING DIFFERENT ELEMENTS

Compound	*Element*	*Ionisation efficiency (C/mole)*	
		Na	*K*
DIP	P	2.2×10^{2}	3.5×10^{2}
Pyridine	N	—	3.7×10^{0}
Triethoxymethylsilane	Si	1.9×10^{0}	6.7×10^{0}
Bromobenzene	Br	1.9×10^{0}	—
Chlorobenzene	Cl	1.1×10^{0}	—
Iodobenzene	I	6.3×10^{-1}	1.6×10^{0}
Thiophene	S	—	-6.0×10^{0}
Tetraethyllead	Pb	-3.7×10^{0}	-4.2×10^{0}
Tetraethyltin	Sn	-8.9×10^{0}	-4.4×10^{1}

TABLE II

RETENTION VALUES OF TIN AND SULPHUR COMPOUNDS AND THEIR STANDARD DEVIATIONS

Compound	*Retention value (mm)*		*σ (mm)*	
	FID	*AFID*	*FID*	*AFID*
Tetraethyltin	116.8	127.6	8.4	14.3
Thiophene	126.2	126.2	15.8	15.9

When following the AFID response to lead and tin compounds, certain anomalies in the shape of the chromatographic curve and retention data of these compounds were observed.

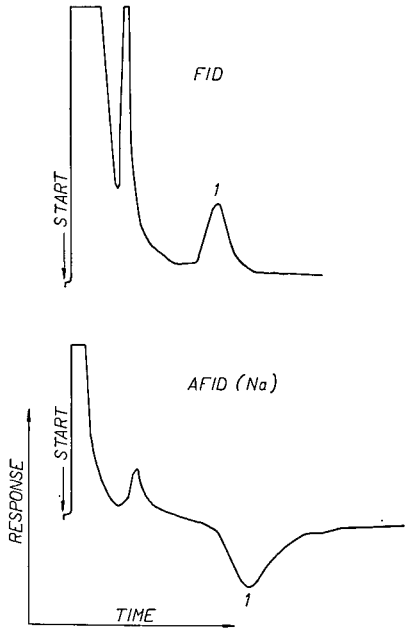


Fig. 3. Chromatograms of tetraethyltin.

The retention data and standard deviations of the chromatographic curves of tetraethyltin with the use of the FID and AFID are presented in Table II. All the chromatographic conditions were identical; in the case of the AFID the tip of the pressed salt of an alkali metal was only fixed to the jet of quartz glass. Both retention value and standard deviation are substantially higher with the AFID than with the FID. Similar differences were also obtained for tetraethyllead. The chromatograms of tetraethyltin with the use of the respective detectors are shown in Fig. 3. Thus it seems that a reaction takes place on the surface of the alkali metal salt. This conclusion is further supported by the fact that a black deposit can be found on the surface of the tip of the pressed alkali metal salt after repeated injections of large amounts of tetraethyllead or tetraethyltin. At the same time the background current decreases. The black deposit disappears after abrasion and the background current again acquires its original value. Another criterion supporting the above conclusion is the fact that the retention curves of lead and tin compounds do not decrease to the original baseline. This effect is very marked at high concentrations of lead and tin compounds.

In order to draw some general conclusions concerning the reaction on the surface of the alkali metal salt in the case of lead and tin, the influence of the use of the tip of the pressed alkali metal salt, *i.e.* comparison of the AFID and FID, was also investigated with respect to the retention volume and standard deviation of the retention curve of thiophene. Thiophene, as a sulphur compound, also has a negative

response in the case of potassium, rubidium and cesium. However, it is obvious from Table II that in this case changes neither in retention value nor standard deviation take place. The reason for the negative response to sulphur compounds apparently differs from the reason for the response to lead and tin compounds.

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1 W. A. AUE, CH. W. GEHRKE, C. R. TINDLE, D. L. STALLING AND CH. D. RUYK, *J. Gas Chromatogr.*, 5 (1967) 381.
2 N. F. IVES AND L. GIUFFRIDA, *J. Ass. Off. Anal. Chem.*, 50 (1967) 1.
3 L. GIUFFRIDA, *J. Ass. Off. Agr. Chem.*, 47 (1964) 293.
4 A. KARMEN, *Anal. Chem.*, 36 (1964) 1416.
5 J. JANÁK, V. SVOJANOVSKÝ AND M. DRESSLER, *Collect. Czech. Chem. Commun.*, 33 (1968) 740.
6 M. DRESSLER AND J. JANÁK, *J. Chromatogr. Sci.*, 7 (1969) 451.
7 M. DRESSLER AND J. JANÁK, *Collect. Czech. Chem. Commun.*, 33 (1968) 3970.
8 M. DRESSLER AND J. JANÁK, *J. Chromatogr.*, 44 (1969) 40.
9 S. LAKOTA AND W. A. AUE, *J. Chromatogr.*, 44 (1969) 472.
10 K. O. GERHARDT AND W. A. AUE, *J. Chromatogr.*, 52 (1970) 47.
11 F. H. POLLARD, G. NICKLESS AND P. C. UDEN, *J. Chromatogr.*, 14 (1964) 1.
12 G. GARZÓ AND D. FRITZ, in A. B. LITTLEWOOD (Editor), *Gas Chromatography 1966*, Inst. Petrol., London, 1967, p. 150.

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CHROM. 5416

A modified pyridine–formic acid gradient for automated column chromatography of amino acids and small peptides

The use of sodium citrate buffers for the separation of amino acids has the disadvantage that before unusual or radioactive amino acids in the eluate can be identified and measured, it is almost invariably necessary to remove the salts. This is time consuming and may result in losses (*e.g.* some sulphur-containing amino acids are oxidised on the ion-exchange resins used for desalting). A volatile buffer system offers obvious advantages[1]. However, the resolution obtained with these buffers is not as good as that obtained with citrate buffers. This paper describes a system that is simple to operate and gives good separation of most of the common amino acids.

Experimental

A single column Technicon amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey) was used. The column (140 cm × 0.6 cm) was packed with Technicon "Chromobeads, Type A" and maintained at 60°. Three buffers (A, B and C) were prepared from pyridine (A.R., British Drug Houses Ltd., Poole, Dorset; purified by redistillation over ninhydrin) adjusted to pH with formic acid (A.R., B.D.H. Ltd., 90 %): (A) pH 2.88, 0.1 *N* pyridine; (B) pH 3.80, 0.1 *N* pyridine and (C) pH 5.00, 1.0 *N* pyridine. Thiodiglycol (0.5 %) was added to buffers A and B, but no detergent was added. The nine-chambered Varigrad was charged according to Table I.

In the trace illustrated in Fig. 1 is represented the analysis of a synthetic mixture containing 0.5 μmole of each amino acid with a buffer flow rate of 1 ml/min of which 0.1 ml/min was passed into the analytical system for reaction with ninhydrin and the remainder was diverted to a fraction collector. The column will, however, tolerate considerably larger amounts of amino acids (2.5–3 μmole) without loss of resolution which is advantageous when minor components of a mixture are being investigated. In the latter case, 0.05 ml/min of the effluent was reacted with nin-

TABLE I

VARIGRAD BUFFER COMPOSITION

Chamber	*MeOH* (*ml*)	*A* (*ml*)	*B* (*ml*)	*C* (*ml*)
1	10	140	—	—
2	6	144	—	—
3	—	150	—	—
4	—	150	—	—
5	—	—	140	10
6	—	12	18	120
7	—	—	—	150
8	—	—	—	150
9	—	—	—	150

hydrin. After each analysis, the column was washed with 0.2 *N* NaOH for 30 min followed by pH 2.88 buffer for 2 h.

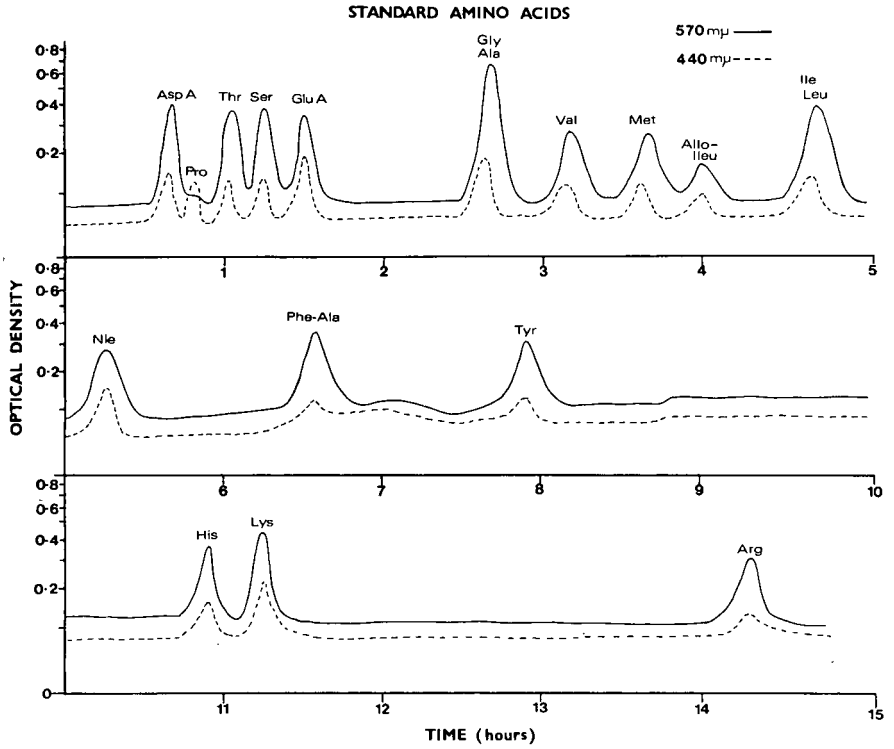

Fig. 1. Chromatogram of a standard mixture of amino acids using the procedure described in the text. The time scale commences 1 h after application of the sample to the column.

Results and discussion

The elution times of some amino acids not shown in Fig. 1 were: hydroxyproline 75 min; S-methylglutathione 65 min; S-methylcysteine 140 min; citrulline 180 min and cystine 400 min. It has not been possible to separate glycine from alanine or isoleucine from leucine by altering the gradient. Indeed, the system is remarkably insensitive to changes in pH in this region. Separation can be effected by lowering the temperature of the column after glutamic acid has emerged, but since the main use of the system is to isolate relatively large amounts of amino acids in a form which facilitates their identification, this is rarely necessary.

Radioactivity in the fractions can be located and measured by scintillation counting, as the buffers give clear solutions when mixed with methyl cellosolve (0.1 ml:2 ml) and scintillator (2,5-diphenyloxazole, 0.5 % in toluene, 10 ml). Although these buffers are supposed to be volatile, in practice it is often difficult to remove the last traces of solvent, especially in early fractions when thiodiglycol is present. Spots applied to paper chromatograms may appear "greasy" and thiodiglycol will

interfere with reagents used to aid identification of sulphur-containing amino acids. Washing the papers in acetone and drying before chromatography removes thiodiglycol and traces of buffer and good chromatograms can be obtained.

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1 P. PADIEU, M. MALEKINA AND G. SCHAPIRA, *3rd Amino Acid Colloquium*, Technicon Instruments Co. Ltd., Chertsey, Surrey, 1965, p. 71.

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CHROM. 5394

Gel chromatography of modified bovine serum on Sepharose

Modified bovine serum (MBS) belongs to one type of blood volume expanders[1–3] which have an important role as infusion material, especially in emergency situations. The preparation of MBS[3,4] investigated in this institute is based on heat denaturation and formolation of a mixture of bovine serum and partially degraded gelatine, followed by partial oxidation by hydrogen peroxide.

MBS represents a complex mixture of molecules and particles of different qualities and sizes. Several methods are used to check the standard physicochemical quality of the product before biological application, *e.g.* electrophoresis[5], viscosimetry[4], gel chromatography on Sephadex G-200[6] and on pearl condensed agar[5,7], etc. However, the latter two techniques served as a rather rough assay of MBS. It is not feasible to fractionate the high-molecular-weight aggregates of molecular weights above about 5×10^5 in MBS on Sephadex G-200 because of the narrow pores of the gel; the pore size of 4% agar pearls was more suitable for this purpose, but the material was neither of standard quality nor chemically homogeneous and the fractionation was not altogether satisfactory.

These disadvantages led us to the use of standard Sepharose gels to investigate whether new information about the distribution of macromolecules present in MBS could be obtained by gel chromatography on this material.

Materials and methods

MBS was a standard preparation (DG 469) prepared in this laboratory. Bead forms of agarose Sepharose 2B, 4B and 6B (Pharmacia, Uppsala, Sweden) were used. The columns were 86 × 1.6 cm for Sepharose 4B and 6B and 80 × 1.4 cm for 2B. 0.9% sodium chloride solution was used as the elution solution. 4% protein solutions

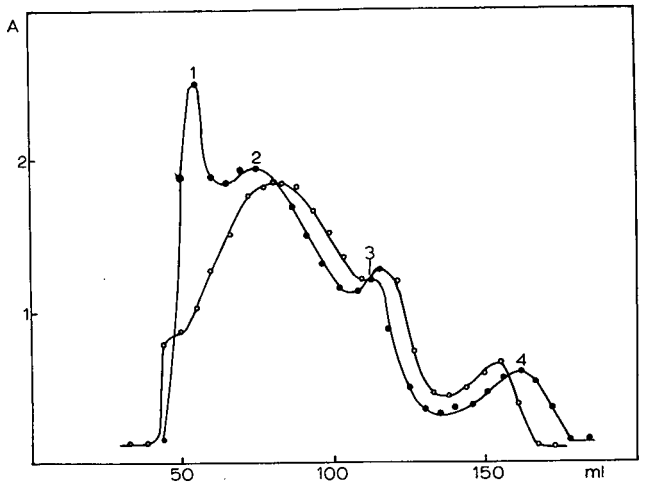

Fig. 1. Gel chromatography of modified bovine serum on Sepharose 4B and 6B. Column, 86 × 1.6 cm; elution solution, 0.9% NaCl. ○, Sepharose 4B; ●, Sepharose 6B.

were applied in 2-ml portions to the top of the column. The flow rate was 5 ml/cm²/h at a temperature of 20–24°. 4-ml fractions were collected and analyzed for proteins by direct photometry at 280 nm.

Results and discussion

The results presented in Fig. 1 indicate that Sepharose 6B and 4B were suitable media for characterizing MBS by four distinct peaks on the elution patterns during gel chromatography. In contrast, Sepharose 2B (Fig. 2) was not suitable since no characteristic fractionation of MBS was achieved, evidently because the sieve structure of this gel was too loose. Chromatography on Sepharose 6B was especially convenient for the analytical check of the standard quality of different batches of MBS. According to the calibration curve based on gel chromatography of standard substances (bovine RNAse, human serum albumin, human γ-globulin and α_2-macroglobulin), a rough estimate of the molecular sizes of the four fractions was made: peak 1 corresponded to proteins having a molecular weight of the order 10^6, peak 2 to about 800,000–1,000,000, peak 3 to about 80,000–100,000 and peak 4 (oxygelatine) to about 10,000–15,000. However, with regard to the differences between the rather compact structure of the calibrating substances and the uncoiled conformation of modified proteins[5–7], the above numbers have only a relative meaning.

The distinct shape of the peaks makes it possible to estimate quantitatively the content and distribution of the four characteristic fractions present in MBS[5,7]. This is very important, especially for the first peak. This fraction consists of the largest aggregates of modified proteins and their condensates with formaldehyde, and its molecular weight is about 10^6. The presence of this fraction seems to be a serious drawback of most MBS preparations because it causes the physical heterogeneity, opalescence and instability of these expanders during storage. It represents also a certain risk to the recipient organism which in some cases might react unfavorably to the infusion of such very high-molecular-weight substances. In this sense, gel

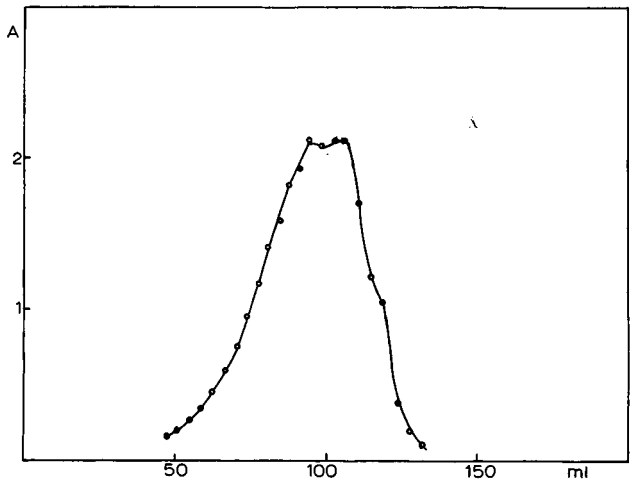

Fig. 2. Gel chromatography of modified bovine serum on Sepharose 2B. Column, 80 × 1.4 cm; elution solution, 0.9% NaCl.

chromatography on Sepharose 6B may be used also as an important means in the search for new methods of preparing MBS of improved quality.

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1 A. G. BORDUAS AND A. FRAPPIER, *Rev. Can. Biol.*, 19 (1960) 395.
2 M. J. PANKRATOVA, *Vopr. Gematol. Perel. Krovi*, 3 (1961) 143.
3 T. I. PŘISTOUPIL AND J. NIKL, *Czech. Pat.*, PV 4562-69 (1969).
4 T. I. PŘISTOUPIL, V.FRIČOVÁ AND E. PALUSKA, *Z. Immunitätsforsch.*, 125 (1963) 50.
5 T. I. PŘISTOUPIL AND S. ULRYCH, *J. Chromatogr.*, 28 (1967) 49.
6 T. I. PŘISTOUPIL, *J. Chromatogr.*, 19 (1965) 64.
7 T. I. PŘISTOUPIL AND S. ULRYCH, *J. Chromatogr.*, 25 (1966) 58.

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CHROM. 5392

Separation of chloroplast pigments on Sephadex LH-20

A method has been developed for separating chloroplast pigments by Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column chromatography. Column chromatographic procedures for the isolation of chlorophylls, carotenes and xanthophylls, using many kinds of adsorbent (*e.g.*, sugar, cellulose powder, magnesia, alumina etc.), have been established for many years[1,2]. However, each of these methods is only effective for a limited number of pigments and cannot be used for a wide range of pigments. For example, the author was unable to obtain a good separation of chlorophyll from its derivatives, *viz.*, pheophytin, pheophorbides and chlorophyllides, with the powdered sugar column chromatography which is reported to be the routine procedure for separating chlorophylls *a* and *b* (ref. 3).

For the purpose of this investigation, the molecular sieve nature of Sephadex LH-20 could not be relied upon to separate these chlorophyll derivatives, because there is only a small difference in molecular weight among these compounds. In fact, when the chromatogram was developed with 80 % methanol instead of chloroform, they were so slightly separated from each other that they appeared as only one broad band. This difficulty could not be overcome by changing the eluent from methanol to acetone, ether, benzene or a mixture of them. DOWNEY *et al.*[4] reported the separation of chloroplast pigments of *Poa tivialis* by means of a Sephadex LH-20 column equilibrated with petroleum ether and eluted with petroleum ether–diethyl ether (80:20). They failed to separate the individual pigments, and β-carotene and pheophytin were eluted as the first band followed by two wide bands of carotenoid and chlorophyll compounds.

It has been reported that the particles of Sephadex LH-20 show a specific affinity towards compounds having a –COOH group, in chloroform[5]. Among the chlorophyll compounds which are commonly observed in plant extracts, those which have a –COOH group are the chlorophyllides and pheophorbides and they seem to be produced by enzymic hydrolysis of chlorophylls and pheophytins, respectively. When laminae of green plants are homogenized with a sufficient amount of magnesium carbonate in the cold, there is no detectable release of chlorophyllides, pheophytins or pheophorbides (except in a few cases such as some tobacco variants[6]).

In this investigation, the potential of Sephadex LH-20 for the separation of chlorophyll compounds and carotenoids was demonstrated and some of the results are briefly reported.

Experimental

Plastid pigments were extracted from the chloroplasts prepared from laminae of *Ambrosia artemisiaefolia* L. according to a method reported previously[7]. The chloroplasts were suspended in 80 % acetone and stirred gently at room temperature under dim light. We had found that the leaves of this plant have a markedly high chlorophyllase activity. Under the above mentioned conditions, the chlorophyllase located in the isolated chloroplasts appears to react on the chlorophyll and organic acids also react on the chlorophyll itself and on its enzymically hydrolyzed products. After 60 min, the suspension was filtered through filter paper and the extraction was

repeated until the residue became colourless. The extracts were combined and transferred to a separating funnel containing a half volume of ethyl ether. After shaking well and separating into two distinct layers, the upper green layer was washed well with 10 % aqueous NaCl solution. The washed ether layer was dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The remaining pigments were dissolved in a small volume of chloroform.

Sephadex LH-20 was swollen in chloroform–methanol (70:30) for 24 h and poured into a column (800 × 20 mm) with stirring to a height of about 60 cm. The suspension medium was replaced with chloroform–methanol (95:5) and finally with chloroform only. When the medium was completely replaced with chloroform the colour of the column changed from white to semi-translucent white and the height of the column bed increased to about 70 cm. After the sample had been applied on the top of the column bed, it was necessary to add a small volume of eluent (chloroform) gradually, because the specific gravity of chloroform is greater than that of the Sephadex particles (sp.gr. = 1.3) and the upper part of the column bed floats at the surface of the eluent. Development with chloroform was continued until the last yellow band was eluted from the column. The second solvent was the mixture chloroform–methanol (97:3); this was used to remove the slow moving green band. The green band remaining at the top of the column bed was developed with chloroform–methanol (70:30). The elution patterns of the chloroplast pigments were monitored spectrophotometrically. Consecutive fractions corresponding to selected regions of the elution diagrams were evaporated to dryness under reduced pressure, and dissolved in 0.5 ml of chloroform. The individual pigments of these pooled fractions were identified by TLC on silica gel or cellulose powder layers. The developing solvents and adsorbents used for TLC are as follows: (1) hexane–acetone (60:40)/silica gel[8]; (2) petroleum ether (60–80°)–acetone–*n*-propanol (90:10:0.45)/cellulose powder[9]; (3) ligroin (100–140°)–chloroform–isopropanol (90:70:10)/silica gel[10].

The separated pigments on the adsorbent layers were detected under UV light and the adsorbent on which the compounds were located was scraped from the glass plate. The compounds were eluted from the adsorbent with appropriate solvents (*i.e.*, ethyl ether for chlorophyll compounds; benzene, chloroform, carbon disulphide and ethanol for carotenoids). The absorption spectra of each pigment were determined in two or three kinds of solvent and compared with those of known pigments[1,2].

Results and discussion

As shown in Fig. 1, the four chlorophyll compounds — chlorophyll, chlorophyllide, pheophytin and pheophorbide — were separated very well. The clear separations observed between chlorophyll and pheophytin, and also chlorophyllide and pheophorbide suggest that the presence or absence of a Mg atom in the molecule causes the difference in the readiness to pass through the molecular sieve of Sephadex LH-20 in chloroform. Chlorophylls *a* and *b* could not be made to emerge as separate peaks. However, the examination of the component of the fractions by TLC indicated that the mobility of chlorophyll *a* was greater than that of *b*, suggesting that it should be possible to obtain a fraction containing a very large proportion of chlorophyll *a* (Fig. 2). Pheophytin and carotene (mainly β-carotene) were eluted from the column in this order, but when the flow rate of the eluent was greater than 2 ml per 10 min, these two compounds were eluted in the same fraction or in the reverse order. The

three orange-yellow compounds were identified as crysanthemaxanthin, auroxanthin and lutein from the top to the bottom. The two other minor compounds could not be identified.

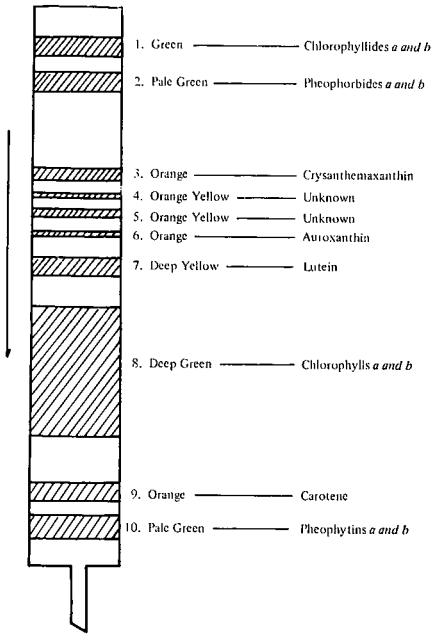

Fig. 1. Chromatographic separation of chloroplast pigments from *Ambrosia artemisiaefolia* L. on a Sephadex LH-20 column equilibrated and eluted with chloroform.

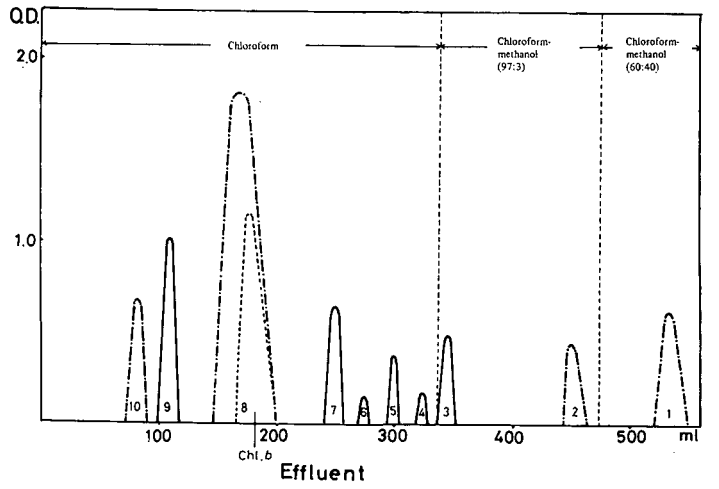

Fig. 2. Fractionation of chloroplast pigments from *Ambrosia artemisiaefolia* L. on a Sephadex LH-20 column equilibrated with chloroform. (–·–·–) absorbance due to carotenoid pigments at 455 mμ. (———) absorbance due to chlorophyll compounds at 655 mμ. Further details are given in the text.

When the period of stirring the chloroplast was prolonged to 10–24 h, three or four green bands were observed between the band of chlorophyll and that of lutein. The compounds corresponding to these bands had absorption spectra which were similar to those of chlorophylls *a* and *b* but clearly differed from them. These chlorophyll compounds seemed to be the chlorophylls *a'* or *b'* of STRAIN[3] or "changed chlorophyll" of BACON AND HOLDEN[9].

It is considered that this method of separating chlorophyll pigments may be suitable for measuring the chlorophyllase activity of plant materials. The enzymic activity has been expressed, in general, in terms of the amount of chlorophylls hydrolyzed (or that of chlorophyllides formed) during the reaction period. Unfortunately chlorophylls and chlorophyllides have the same absorption spectrum[11]; therefore in order to determine the amount of the latter spectrophotometrically, it is necessary to separate these two compounds completely. This has been carried out on the basis of the differences in their solubility towards organic solvents and their mobility on paper chromatograms[7]. These procedures, however, are of questionable accuracy in quantitative terms and troublesome to carry out. The present method is therefore considered to be particularly appropriate for this purpose. In fact, it seemed to be better to use a mixture of chloroform and methanol (95:5) as eluent instead of chloroform and also a shorter column (about 10 cm) for measuring chlorophyllase activity. Development with this chloroform–methanol mixture gave a rather incomplete separation of xanthophylls, but the time consumed in separating the chlorophylls from their enzymic products and eluting the latter from the column was shortened to a certain degree.

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1 M. HOLDEN, in T. W. GOODWIN (Editor), *Chemistry and Biochemistry of Plant Pigments*, Academic Press, London, 1965, p. 462.
2 B. H. DAVIES, in T. W. GOODWIN (Editor), *Chemistry and Biochemistry of Plant Pigments*, Academic Press, London, 1965, p. 489.
3 H. H. STRAIN, in *Chloroplast Pigments and Chromatographic Analysis, 32nd Annual Priestley Lectures*, Penn. State Univ., Univ. Park, Pa., 1958.
4 W. K. DOWNEY, R. F. MURPHY AND M. K. KEOGH, *J. Chromatogr.*, 46 (1970) 120.
5 *Technical Bulletin of Sephadex LH-20*, Pharmacia Fine Chemicals, Uppsala, Sweden, 1966.
6 S. SHIMIZU, R. HOTTA AND E. TAMAKI, *Bot. Mag. (Tokyo)*, 79 (1966) 560.
7 S. SHIMIZU AND E. TAMAKI, *Bot. Mag. (Tokyo)*, 75 (1962) 462.
8 C. P. BOND, *J. Sci. Food Agr.*, 18 (1967) 161.
9 M. F. BACON AND M. HOLDEN, *Phytochem.*, 6 (1967) 193.
10 E. KESSLER AND F. C. CZYGAN, *Ber. Deut. Bot. Ges.*, 78 (1966) 342.
11 S. SHIMIZU AND E. TAMAKI, *Arch. Biochem. Biophys.*, 102 (1963) 152.

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CHROM. 5404

Effects of pesticide-grade hexanes on the silicic acid chromatography of polychlorinated biphenyls and organochlorine pesticides

Small amounts of aromatic hydrocarbons such as benzene, present in some commercial pesticide-grade hexanes, affect significantly the elution patterns of polychlorinated biphenyls (PCB) and organochlorine pesticides from silicic acid columns.

Chromatography on silicic acid has been used to clean up extracts in the determination of PCB and organochlorine pesticides[1,2]. The quality of pesticide-grade hexane is specified at best by its boiling range, and, as shown below, the results will not be reproducible when different pesticide-grade hexanes are used. It is likely that the discrepancies in the elution patterns of PCB and organochlorine pesticides from Florisil columns, reported in the literature[3,4], were observed because different pesticide-grade hexanes were used.

Experimental

Silicic acid Silicar® 100–200 mesh was activated at 130° overnight and deactivated by the addition of 3 % water as described[2]. The chromatography was carried out using 2 g of Silicar® in 45 × 0.7 cm glass columns. PCB (Aroclor® 1254) and pesticides were applied to the columns in 1.5 ml of hexane, washed into the columns with an additional 1.5 ml of hexane and the columns were eluted with hexane, collecting 10 ml (fraction I) and 20 ml (fraction II) of the effluent, and with 10 ml of 10 % diethylether in hexane (fraction III). The mixture of pesticides applied to the columns contained hexachlorobenzene (C_6Cl_6), lindane, heptachlor, aldrin, heptachlor epoxide, *p,p'*-DDE, dieldrin, *p,p'*-DDD, and *p,p'*-DDT in amounts of 6.9, 46.5, 46.5, 48.0, 90.6, 121.0, 64.8, 136.0, and 136.0 ng, respectively. The amount of Aroclor® 1254 used was 689 ng. Average recovery of the pesticides and PCB was 92 %.

A Packard A7901 gas chromatograph with a glass column (6 ft. × 4 mm), containing 4 % SE-30 on acid-washed Chromosorb W, 100–120 mesh, operated at 200°, was used. Carrier gas was nitrogen at a flow rate of 60 ml/min. Injector and detector were kept at 210°, D.C. voltage in the electron-capture detector was 80 V and the meter sensitivity for PCB and organochlorine pesticides was 3×10^{-9} and 1×10^{-8} A, respectively. Peak area determined by a disc integrator was used to quantify PCB and all pesticides except hexachlorobenzene, where peak height was used.

Pesticide-grade hexanes were supplied by the Fisher Scientific Company and by Matheson Coleman & Bell. UV spectra of both hexanes, diluted 1 to 10 with Fisher Scientific Company spectrograde hexane, were recorded on a Beckman DK2A spectrophotometer.

Results and discussion

Benzene absorption peaks at 259, 253.5, and 247.5 nm were observed in both hexanes. The absorbance increased sharply to values > 1 at shorter wavelengths in the Fisher Scientific Company hexane, while benzene peak at 242 nm was observed in the Matheson Coleman & Bell hexane. Based on the absorbance at 259 nm, the former and the latter contained 0.79 and 0.21 ml/l of benzene, respectively. Pesticide-grade

TABLE I

CHROMATOGRAPHY OF AROCLOR® 1254 AND ORGANOCHLORINE PESTICIDES ON SILICIC ACID

H = heptachlor, A = aldrin. Hexachlorobenzene was always eluted in fraction I, lindane, heptachlor epoxide, dieldrin, and *p,p'*-DDD were always eluted in fraction III.

Hexane	*Benzene added (ml/l)*	*% in fractions*[a]				
		Aroclor®	*H*	*A*	*p,p'-DDE*	*p,p'-DDT*
Fisher Scientific	—	56 (I)	100 (II)	71 (I)	100 (II)	100 (III)
Matheson Coleman & Bell	—	18 (I)	75 (II)	22 (I)	56 (II)	100 (III)
Matheson Coleman & Bell	5	69 (I)	100 (II)	16 (I)	100 (II)	100 (III)
Matheson Coleman & Bell	10	64 (I)	100 (II)	80 (I)	100 (II)	100 (III)
Matheson Coleman & Bell	20	86 (I)	33 (I)	100 (I)	31 (I)	68 (II)

[a] Balance eluted in the next fraction

hexane supplied by the Fisher Scientific Company elutes 56 % of Aroclor® 1254 in fraction I and 100 % of *p,p'*-DDE in fraction II. With pesticide-grade hexane from Matheson Coleman & Bell, only 18 % of Aroclor® 1254 and 56 % of *p,p'*-DDE appear in fractions I and II, respectively (Table I). The addition of benzene (5–10 ml/l) to the Matheson Coleman & Bell hexane makes the elution patterns practically identical with those of the Fisher Scientific Company hexane.

It is therefore necessary to characterize in more detail any commercial hexane used in the chromatography of PCB and organochlorine pesticides on silicic acid and possibly also on other adsorbents.

The most pronounced difference between the PCB peaks present in fractions I and II was the complete absence of peaks with relative retention times of 0.97 and 1.04 (*p,p'*-DDE = 1.00) from the fraction I. It is also worth noticing that hexachlorobenzene (C_6Cl_6) appears always in fraction I and lindane in fraction III. Since these two compounds have very similar retention times on gas chromatography, their separation on silicic acid is a useful confirmation technique.

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1 A. V. HOLDEN AND K. MARSDEN, *J. Chromatogr.*, 44 (1969) 481.
2 J. A. ARMOUR AND J. A. BURKE, *J. Ass. Offic. Anal. Chem.*, 54 (1970) 761.
3 L. M. REYNOLDS, *Bull. Environ. Contam. Toxicol.*, 4 (1969) 128.
4 A. BEVENUE AND J. N. OGATA, *J. Chromatogr.*, 50 (1970) 142.

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CHROM. 5386

Enzymatic and polarographic determination of Metation and its analogues after separation on silica gel thin layers

It is possible to determine polarographically a mixture of Metation (O,O-dimethyl-O-(4-nitro-3-methylphenyl)-phosphothioate) and its analogues (Metaoxon: O,O-dimethyl-O-(4-nitro-3-methylphenyl)-phosphate; S-methyl isomer, bis-(*p*-nitro-methylphenyl) isomers) after its separation on silica gel layers[1]. Moreover, various substrates and enzymatic sources of esterases have been used[2–8] for the estimation of the inhibition properties of cholinesterase inhibitors.

In order to make more effective use of the samples in the analysis of organophosphate insecticides we combined these two analytical steps, *viz.* the enzymatic determination of the inhibition effect of Metation on esterases and its polarographic determination.

Apparatus

All polarographic measurements were performed on a Polarograf LP-60 with an EZ-2 recorder (product of Laboratorní přístroje, Prague) connected to it. Kalousek's vessel with a saturated calomel electrode was used. When the height of the mercury reservoir was 70 cm, the capillary used had an out-flow velocity of 2.3 mg/sec and a drop time of 3.1 sec.

Solutions and reagents

A solution of $3.61 \cdot 10^{-3}$ *M* Metation (VÚAgT, Bratislava) and, $3.84 \cdot 10^{-3}$ *M* Metaoxon in chloroform were used. Sörensen's borate buffer was pH 9.2; the enzymatic source was a homogenate of beef liver[2]; the visualisation reagent was *β*-naphthyl acetate with Fast Blue B[2]. A 1 % solution of gelatine was also used.

Procedure and construction of the calibration curve

10–40 *μ*l of a stock solution of Metation are applied onto a silica gel layer and developed in a mixture of light petroleum (b.p. 60–80°)–acetone (3:1. After finishing the development and drying the silica gel layer the chromatographic plates are sprayed with enzyme solution and are allowed to incubate at 37° in a water-saturated chamber for 30 min. The plates are oversprayed with the visualisation reagent and semiquantitative evaluation of the inhibition efficiency is planimetrically calculated from the areas corresponding to the spots (see Fig. 1)[6]. The spots containing the separated substances are transferred into the chromatographic columns and are eluted with 4 ml of acetone and collected in 10-ml volumetric flasks. 0.1 ml of gelatine solution is added and the volumetric flasks are made up with borate buffer. These solutions are transferred to a Kalousek's vessel and after desoxygenation with nitrogen the polarographic waves are recorded from —0.4 V to —0.8 V (see Fig. 2).

Discussion and conclusions

The advantage of the method described lies in the possibility of analysing even very small quantities of samples due to their better utilisation.

The contribution of each substance separated to the whole inhibition effect can

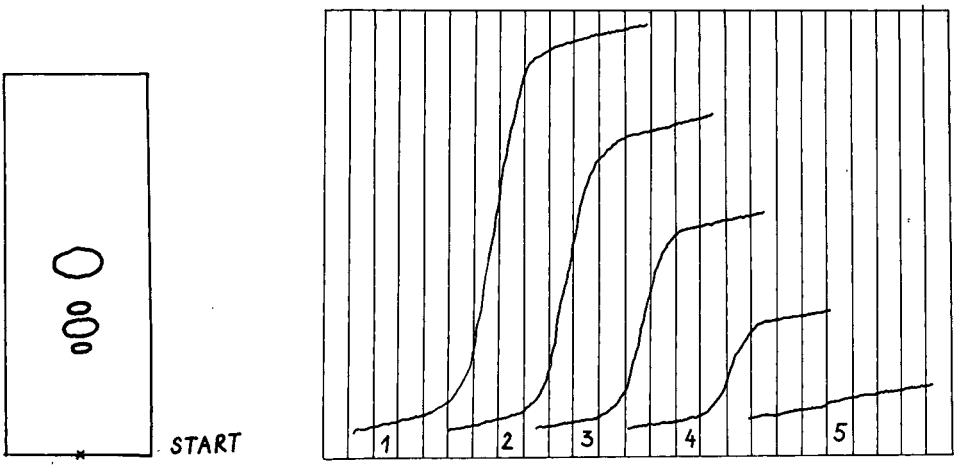

Fig. 1. Separation of Metation from its analogues. Silica gel/light petroleum (b.p. 60–80°)–acetone (3:1), enzymatic visualisation. From origin: spot 1, Metaoxon; spots 2 and 3, isomers of Metation; spot 4, Metation.

Fig. 2. Polarogram for the construction of the calibration curve for Metation after elution from thin layers. (1) 4 μg/ml; (2) 3 μg/ml; (3) 2 μg/ml; (4) 1 μg/ml; (5) 0 μg. Sensitivity 1:5, performed according to the working procedure described in the text.

be calculated after the chromatographic separation of Metation and its analogues and the planimetric semiquantitative evaluation of their inhibition efficiency. The actual percentage composition of the samples analysed is determined polarographically from the same plates.

The presence of unreacted β-naphthyl acetate and Fast Blue B and compounds arising from the coupling of enzymatically released β-naphthol with Fast Blue B does not disturb the subsequent polarographic determination of the substance to be analysed after its elution from the thin layer of silica gel.

The standard deviation of the semiquantitative enzymatic determination is ±20%, of the polarographic determination ±6%.

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1 J. KOVÁČ, *J. Chromatogr.*, 11 (1963) 412.
2 W. P. MCKINLEY AND P. S. JOHAL, *J. Ass. Off. Agr. Chem.*, 46 (1963) 840.
3 S. I. READ AND W. P. MCKINLEY, *J. Ass. Off. Agr. Chem.*, 46 (1963) 863.
4 P. J. BUNYAN, *Analyst*, 89 (1964) 615.
5 S. P. COLOWICK AND N. O. KAPLAN, *Methods Enzymol.*, 1 (1955) 642.
6 W. WINTERLIN, G. WALKER AND H. FRANK, *J. Agr. Food Chem.*, 16 (1968) 808.
7 P. J. WALES, C. E. MENDOZA, H. A. MCLEOD AND W. P. MCKINLEY, *Analyst*, 93 (1968) 691.
8 C. E. MENDOZA, D. L. GRANT, B. BRACELAND AND K. A. MCCULLY, *Analyst*, 94 (1969) 805.

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CHROM. 5433

The use of chloroplatinic acid as a chromatographic spray for sulfur compounds

This report describes an innovation on the use of chloroplatinic acid as a chromatographic spray reagent in the detection of some sulfur compounds.

In the course of our studies on the occurrence of bitterness in chopped onions, (*Allium cepa*), we obtained extracts from onions which contained sulfur-bearing compounds other than those responsible for the typical onion odor and flavor. Although these crude extracts have been resolved into a number of different fractions by adsorption chromatography, the identities of the sulfur compounds have not yet been completed. The proof of the presence of these sulfur compounds, however, has been established by elemental analyses.

In order to monitor the eluates from the separation columns, we found it expedient to use thin-layer chromatography (TLC) because of the advantage of speed. This was accomplished by ascending solvent techniques on ½ in. × 8 in. glass strips coated with silicic acid containing 5 % rice starch as binder. Demonstration of the presence of the sulfur compounds was done by spraying with a sulfur-indicating reagent.

Because of the unidentified status of these compounds, one could not readily select a particular sulfur-reactive reagent with any degree of certainty that the desired result would be obtained. A number of reagents including FEIGL's reagent[1], its variation by CARSON AND WONG[2], the iodine–azide method of CHARGAFF *et al.*[3], as well as the iodoplatinate procedure of WINEGARD *et al.*[4] and the variation described by TOENNIES AND KOLB[5] were tried.

In our hands, the reagent of TOENNIES AND KOLB[5] showed the most promise. The spray reagent finally adopted for use was made up according to the following formula: 14.5 mg Chloroplatinic acid, 311 mg KI, 2.8 ml 2 *N* HCl and absolute ethanol to 500 ml volume. The reagent is stable for several months if kept refrigerated. On the TLC strips, the presence of the sulfur compounds was indicated by a rarefied or faintly bleached area against a tan to light brown background. While the results showed consistency they were often difficult to observe, especially if the concentrations of the unknowns were low. Fuming with HCl vapors as suggested by WINEGARD *et al.*[4] and TOENNIES AND KOLB[5] did not improve the contrast between the background and reaction sites.

However, a striking change takes place if the TLC strips, after having been sprayed with the chloroplatinic acid reagent, are dried and then re-sprayed with distilled water. The brownish background quickly turns to a purple color, and the here-to-fore bleached areas of low contrast become white in sharp relief. We make no attempt to explain the phenomenon. However, the presence of the starch binder in the TLC coating is necessary for this change to occur. This sharpness in contrast is maximal after the TLC coating dries. The color effect is stable for several days; but once faded, it can be restored by a light re-spraying of distilled water. Fig. 1 shows the degree of this contrast between the water sprayed and the untreated TLC strips developed with the chloroplatinic acid reagent.

The sensitivity obtained by use of this procedure on some sulfur-bearing compounds is shown in Table I. It may be noted here that in comparison with some of the sulfur reagents mentioned earlier in this paper, particularly with regard to cysteine, cystine, and methionine; the iodine–azide method of CHARGAFF *et al.*[3] requires a larger sample (5 ×) and also considerable development time (up to 60 min for methionine). On the other hand, while the color development of the iodoplatinate reagent of WINEGARD *et al.*[4] is in the order of rapidity of the method described in this paper, quantities necessary for detection are reported to be approximately twelve times the amounts detectable with the chloroplatinic acid; and, in the case of methionine sulfoxide and methionine sulfone, even higher concentrations are necessary.

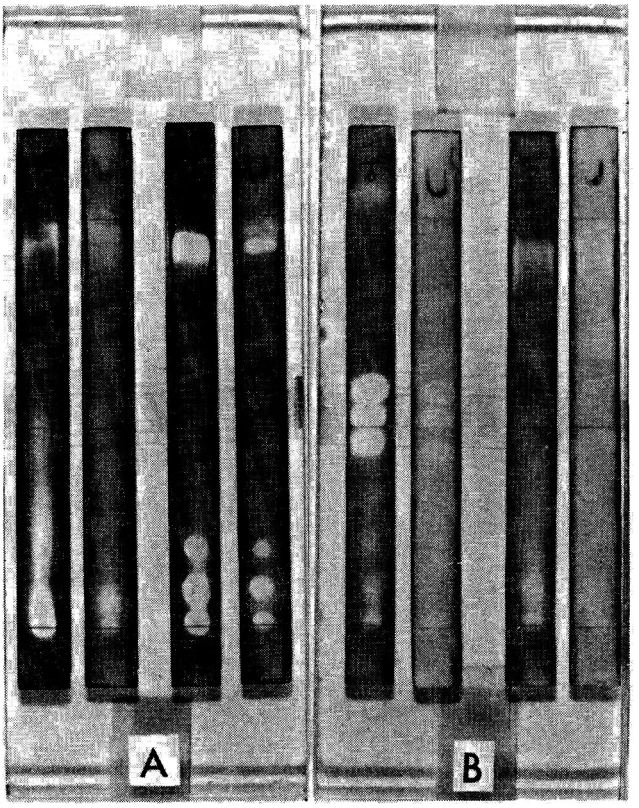

Fig. 1. Contrast obtained with TLC strips (left strip of each pair) after re-spraying with water. (A) Shortly after re-spraying, (B) 24 h later.

The effectiveness of the novel use of the water spray is also applicable to paper chromatograms of these sulfur compounds, providing that a starch-treated paper is used for the chromatography. We have found that dipping papers such as Whatman No. 1 or S & S No. 589 Blue Ribbon, for example, in a commercial soluble starch solution diluted with four parts water and air-dried in a fume hood prior to use was

convenient. After development of the chromatograms, these were handled in the same manner as the TLC strips. With the papers, the change in background color is from a pale pink to a deep violet.

TABLE I

SENSITIVITIES OBTAINED[a] USING CHLOROPLATINIC ACID REAGENT ALONE AND WITH RESPRAYING OF TLC STRIPS

Sulfur compound	*Reagent only*	*Re-sprayed*	*Sulfur compound*	*Reagent only*	*Re-sprayed*
Cysteine·HCl·H_2O	1–3	0.5	Methionine sulfone	5	1
L-Cystine	1	1	S-Benzyl-L-cysteine	1	0.5[b]
Methionine	1–2	1[b]	Cycloalliin	0.5	0.5
Methionine sulfoxide	1–3	0.5[b]	6-Mercapto succinic acid	0.5	0.5
S-*n*-Propyl cysteine-sulfoxide	0.5	0.5[b]	Thiourea	0.25	0.25

[a] Sensitivity expressed as p.p.m. (wt./vol.).
[b] Dark purple or purple spot with white halo against background.

TABLE II

RESULTS OBTAINED BY RE-SPRAYING WITH WATER, CHROMATOGRAMS DEVELOPED WITH CHLOROPLATINIC ACID REAGENT

Sulfur compound	*TLC strips*[a]	*Starch treated paper*[b]
Cysteine·HCl·H_2O	Very strong white	Strong white
L-Cystine	Very strong white	Strong white
Methionine	Purple spot with white halo	Light purple[c]
Methionine sulfoxide	Purple spot with white halo	Weak white[c]
S-*n*-Propyl cysteinesulfoxide	Purple spot with white halo	Weak white[c]
Methionine sulfone	Pale white	Pale white
S-Benzyl-L-cysteine	Purple spot with white halo	Weak purple[c]
Cycloalliin	Purple spot with white halo	Weak purple[c]
6-Mercapto succinic acid	Very strong white	Very strong white
Thiourea	Very strong white	Very strong white
Methyl sulfide	Strong white	Strong white
n-Propyl sulfide	Strong white	Faint purple[c]
Methyl disulfide	Weak white	Weak purple[c]
n-Propyl disulfide	Strong white	Weak purple[c]

[a] Background color is deep purple.
[b] Background color is deep violet.
[c] Fades on standing, but quickly restored by light re-spraying with water.

This procedure has been tested with some known sulfur-bearing compounds on both TLC strips and paper chromatograms. The results obtained are shown in Table II. In most of the examples showing weak responses, the compounds being tested either showed no observable reaction or were barely discernible in the media not re-sprayed with water. The aliphatic mono- and disulfides were included as spot tests to show the effectiveness of this method with these very volatile compounds.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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1 F. FEIGL, *Spot Tests in Organic Analysis*, 6th Ed., Elsevier, Amsterdam, 1960.
2 J. F. CARSON AND F. F. WONG, *J. Chromatogr.*, 12 (1963) 408.
3 E. CHARGAFF, C. LEVINE AND C. GREEN, *J. Biol. Chem.*, 175 (1948) 67.
4 H. M. WINEGARD, G. TOENNIES AND R. J. BLOCK, *Science*, 108 (1948) 506.
5 G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823.

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CHROM. 5385

Thin-layer chromatography and elution of picogram amounts of estradiol

The recent development in protein-binding methods (also called saturation analysis[1]) has made it possible to determine picogram amounts of estradiol (E_2, estra-1,3,5(10)-triene-3,17β-diol) in biological samples utilizing anti-estradiol antibodies[2–4] or receptor proteins from the uterus of various species[5,6]. Although these proteins are highly specific, at least one chromatographic step is usually required to separate E_2 from a few crossreacting estrogens and from some unknown material which interferes with the endpoint determination. This study was initiated during the development of a hapten-radioimmunoassay[7]. We realized that it is not possible to conclude that a chromatographic system and elution procedure which will permit quantitative recoveries in the microgram range, will also be reliable when applied to nano- or picogram amounts of that particular compounds. Therefore conditions were developed which allow artifact free thin-layer chromatography (TLC) and elution of picogram amounts of E_2 and yield blank values in the endpoint determination by a hapten-radioimmunoassay system below 10 pg.

Materials

All reagents were analytical grade and purchased from Merck AG Darmstadt, unless otherwise stated. Ethanol, 2-mercaptoethanol (puriss. Serva, Heidelberg), phenol and naphthalene were used without further purification. Benzene and ethyl acetate were distilled through a Vigreux column. Methanol was redistilled after treatment with 2,4-dinitrophenylhydrazine[8]. Dichloromethane was treated with 0.1 vol. conc. sulfuric acid for a few days, washed once with 0.1 vol. 2 *N* NaOH, then three times with 0.1 vol. demineralized water, dried over anhydrous sodium sulfate and distilled. The purity of estradiol-17β was checked by TLC using the solvent systems benzene–ethyl acetate (75:25) and benzene–ethanol (90:10). Estradiol-17β-6,7-^{3}H (40 Ci/mmole) and estradiol 17β-2,4,6,7-^{3}H (107 Ci/mmole) were obtained from New England Nuclear Corp. A purity greater than 95 % was established. For this purpose 3 μg unlabeled and 100,000 d.p.m. labeled estradiol were spotted on TLC plates and developed in the systems already mentioned. After scanning (Dünnschicht-Scanner II, Berthold/Frieske GmbH, Karlsruhe-Durlach), the estradiol peak and the remaining radioactivity were eluted separately with methanol into counting vials, and after evaporation and addition of a toluene based scintillator the radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

The thin-layer plates, 20 × 20 cm, precoated with Silica Gel F254 (Merck AG, Darmstadt, catalog number 5715/0025), were purified by ascending chromatography in methanol three times, air dried for 1/2 h and stored in a tight case specially designed for this purpose (Desaga, Heidelberg). All glassware used was cleaned with a detergent, rinsed with tap water and demineralized water. Nitrogen, purified, was obtained from LINDE AG, München-Lohhof, methane from the laborabory Prof. Dr. BERTHOLD, Wildbad.

Methods

Standard procedure. 100,000 d.p.m. of E_2-6,7-^{3}H (total mass 306 pg) dissolved in 1 μl benzene containing 10 % ethanol were spotted four times on the starting line of the TLC plate at a distance of 4 cm from each other. On each side 5 μg E_2 were placed as a marker. One or two plates were placed in a tank lined with filter paper and saturated for at least 2 h with the solvent system benzene–ethyl acetate (75:25). After the solvent front had reached the 15 cm line the plates were taken out for 3–5 min and the chromatography was then repeated as before. The first chromatography is called Ia, the second one Ib. After localisation of the marker spots by UV absorbtion the corresponding areas of the labeled E_2 (2 × 3 cm) were scraped off with an adapted razor blade and sucked into a Pasteur pipette plugged with glass wool. The E_2 was eluted three times with 1 ml of 30 % methanol in dichloromethane into small culture tubes. After evaporation in nitrogen at 40° the residues were spotted on TLC plates using about 100 μl 10 % methanol in dichloromethane three times. Again the chromatogram was developed twice in the system benzene–ethyl acetate (75:25) (chromatography IIa and IIb).

Variations of the standard procedure. (1) Instead of 306 pg of labeled E_2 (corresponding to 100,000 d.p.m. E_2-6,7-^{3}H, 40 Ci/mmole) 81 and 40.5 pg (corresponding to 70,600 and 35,300 d.p.m. of E_2-2,4,6,7-^{3}H, 107 Ci/mmole) were used.

(2) 100,000 d.p.m. E_2-6,7-^{3}H (40 Ci/mmole) were mixed with various amounts of unlabeled E_2 (3 μg, 500 ng, 50 ng, 5 ng, 0.5 ng), chromatographed, eluted, and rechromatographed as described.

(3) The interval between the end of chromatography Ib and the start of chromatography IIa was varied. The plates, protected only from sunlight, were exposed to the air in the laboratory before elution was started.

(4) The benzene–ethyl acetate system was modified by adding: (a) 100 mg phenol; (b) 100 μl 2-mercaptoethanol; or (c) 100 mg naphthalene to 100 ml of the solvent mixture.

(5) Instead of the benzene–ethyl acetate system benzene–ethanol (90:10) was used.

(6) TLC plates spotted only with the marker E_2 were chromatographed in the benzene–ethyl acetate system containing either phenol or 2-mercaptoethanol. At the height of the marker E_2 areas (2 × 3 cm) were scraped off and eluted as described. The residues after evaporation were tested as blank values in a hapten-radioimmunoassay system.

Results

Fig. 1 (lanes 1 and 2) demonstrates the extensive artifact formation in the case that only 300 pg E_2-6,7-^{3}H (40 Ci/mmole) are chromatographed, eluted, and rechromatographed as described under *Standard procedure*. The E_2 peak is incompletely separated from the degradation products, some of which are less polar and some more polar than E_2 in that particular chromatographic system. No attempt has been made to identify these products. By adding 3 μg unlabeled E_2 to 300 pg labeled E_2 artifact formation becomes negligible (lanes 3 and 4). If instead of the benzene–ethyl acetate system benzene–ethanol (90:10) was used the degree of artifact formation was

essentially the same, but the chromatographic behavior of the degradation products was slightly different.

The degree of artifact formation as a function of the total mass of E_2 is shown in Fig. 2 (lane 1: 300 pg labeled E_2 + 0.5 ng unlabeled E_2; lane 2: + 5 ng E_2; lane 3: + 50 ng E_2; lane 4: + 500 ng E_2. It can be seen that 500 ng E_2 are required to prevent artifact formation.

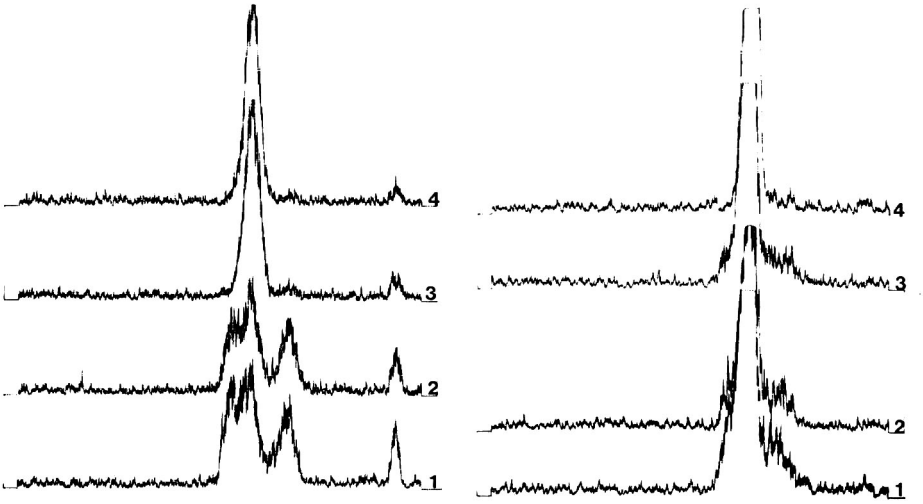

Fig. 1. Radiochromatogram after TLC, elution, and a second TLC as described (*Standard procedure*). Lanes 1 and 2, 300 pg tritiated E_2; lanes 3 and 4, 300 pg tritiated E_2 + 3 μg unlabeled E_2.

Fig. 2. Chromatography, elution, and rechromatography of 300 pg tritiated E_2 + 0.5 ng unlabeled E_2 (lane 1), + 5 ng E_2 (lane 2), + 50 ng E_2 (lane 3), + 500 ng E_2 (lane 4).

Fig. 3 illustrates that the longer E_2 is adsorbed onto the silica gel (exposed to the air at room temperature) the greater is the extent of artifact formation. Each time 300 pg labeled E_2 were used according to the standard procedure. In case of lane 1 working up the sample between the end of chromatography Ib and the start of chromatography IIa was performed as rapidly as possible and took about 10 min. For lane 2 this interval was 1 h; for lane 3, 5 h; for lane 4, 18 h.

Fig. 4 demonstrates that artifact formation on the TLC plates is prevented by adding a small amount of a mild reducing agent to the chromatographic system. Lane 1 is the chromatogram of the control experiment performed simultaneously. The time between the end of chromatography Ib and the start of chromatography IIa was 1 h, all the other details were identical with the standard procedure. Lane 2: This experiment differs from the control experiment only in the fact that 100 mg phenol were added to 100 ml of the solvent system. Lane 3: Phenol was replaced by 100 μl 2-mercaptoethanol. Lane 4: Phenol was replaced by 100 mg naphthalene. The last experiment confirms that the protective effect of phenol and mercaptoethanol is due to their reducing properties.

The experiments summarized in Fig. 4 were repeated with 80 and 40 pg E_2-2,4,6,7-^{3}H (107 Ci/mmole). The chromatograms are essentially the same as those

shown in Fig. 4, indicating that amounts as small as 40 pg E_2 can be chromatographed without artifact formation.

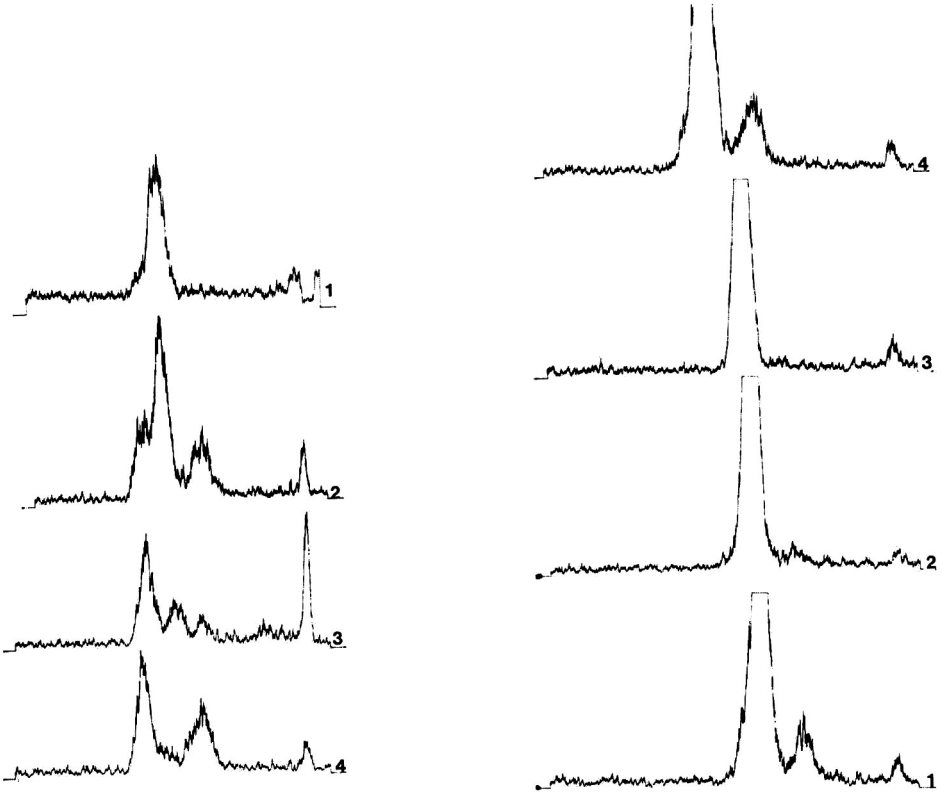

Fig. 3. Chromatography, elution, and rechromatography of 300 pg tritiated E_2. The interval between the end of chromatography Ib and the start of chromatography IIa was 10 min (lane 1); 1 h (lane 2); 5 h (lane 3); 18 h (lane 4).

Fig. 4. Lane 1: control chromatogram as in Fig. 1, lanes 1 and 2. Lane 2: the benzene–ethyl acetate system was modified by adding 100 mg phenol per 100 ml solvent mixture. Lane 3: phenol is replaced by 100 μl 2-mercaptoethanol. Lane 4: phenol is replaced by 100 mg naphthalene.

The blank values of TLC plates after development in a system containing either phenol or mercaptoethanol were tested in a hapten-radioimmunoassay system (see *variation of the standard procedure* (6)) and were found to be constantly below 10 pg.

Discussion

The experiments reported demonstrate that under the specified conditions amounts as small as 40 pg tritiated E_2 survive chromatography on silica gel thin-layer plates, elution, and evaporation. It is assumed that the unlabeled compound is also stable under these conditions. The artifact formation depended on the amount of E_2 per surface area as well as on the duration of the adsorbtion of E_2 on the silica gel. Since the presence of reducing agents throughout the whole procedure could prevent the formation of artifacts, they were obviously caused by oxygen. There is no cogent reason to conclude that atmospheric contaminants are responsible for

this kind of artifact as has been claimed[9]. Since impurities in the solvents are known to cause degradation of various steroids during evaporation[10,11], especially in the presence of silica gel particles[12–15], we only used solvents of high purity.

We did not evaluate to what extent phenol or mercaptoethanol are removed after evaporation in nitrogen at 40°, since the blank values in the endpoint determination by a hapten–radioimmunoassay system were below 10 pg. If another kind of endpoint determination is to be used, the procedure has to be reconsidered.

It is possible that other compounds could also be protected from degradation during TLC if the principle involved in our procedure is utilized for this purpose.

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1 R. P. EKINS, G. B. NEWMAN AND J. L. H. O'RIORDAN, in R. L. Hayes, F. A. GOSWITZ AND B. E. P. MURPHY (Editors), *Radioisotopes in Medicine: In Vitro Studies*, U. S. Atomic Energy Commission, Oak Ridge, 1968, p. 59.
2 G. E. ABRAHAM, *J. Clin. Endocrinol.*, 29 (1969) 866.
3 A. R. MIDGLEY, Jr. and G. D. NISWENDER, *Acta Endocrinol. Suppl.*, 147 (1970) 320.
4 G. MIKHAIL, C. H. WU, M. FERIN AND R. L. VANDE WIELE, *Acta Endocrinol. Suppl.*, 147 (1970) 347.
5 S. G. KORENMAN, L. E. PERRIN AND T. P. MCCALLUM, *J. Clin. Endocrinol.*, 29 (1969) 879.
6 D. A. SHUTT, *Steroids*, 13 (1969) 69.
7 P. DOERR, to be published.
8 I. E. BUSH, *The Chromatography of Steroids*, Pergamon, Oxford, London, 1961, p. 348.
9 J. COYOTUPA, K. KINOSHITA, R. Y. HO, C. CHAN, W. PAUL, M. FOOTE AND S. KUSHINSKY, *Anal. Biochem.*, 34 (1970) 71.
10 B. P. LISBOA, *Methods Enzymol.*, 15 (1969) 154.
11 A. I. FRANKEL AND A. V. NALBANDOV, *Steroids*, 8 (1966) 749.
12 D. R. IDLER, N. R. KIMBALL AND B. TRUSCOTT, *Steroids*, 8 (1966) 865.
13 D. R. IDLER AND D. A. HORNE, *Steroids*, 11 (1968) 909.
14 J. ATTAL, S. M. HENDELES, J. A. ENGELS AND K. B. EIK-NES, *J. Chromatogr.*, 27 (1967) 167.
15 F. A. VANDENHEUVEL, *J. Lab. Clin. Med.*, 69 (1967) 343.

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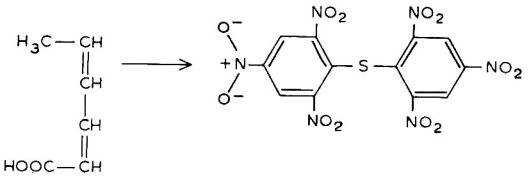
CHROM. 5363

Studies on charge transfer complexes of some conjugated compounds with explosives

According to DEWAR's molecular orbital theory[1] conjugated compounds are characterised as π donors. It is likely that in nature compounds such as sorbic acid, vitamin A, α-, β-, and γ-carotenes, lycopene, β-citraurin etc., function as $\pi\pi$, $n\pi$ or similar charge transfer complexes. Sorbic acid forms a complex with diaminooxidase[2]. Its inhibitory effect on the respiration rate in the cell is due to the formation of a sorbic acid–coenzyme A complex[3]; a vitamin A–rhodopsin complex is known[4]. Vitamin A and the hydrocarbon carotenoids exist as protein complexes and are transported by the serum lipoprotein[5–7]. Vitamin A in rats, as in man, is associated with α_1-globulin[8].

Although such compounds have been separated by thin-layer chromatographic (TLC) techniques, no work is reported where they have been demonstrated as π donors to nitro aromatic compounds, which are excellent π acceptors.



Sorbic acid–2,4,6,2′,4′,6′-hexanitrodiphenyl sulphide complex.

The present paper describes the resolution and identification of some conjugated compounds as π complexes with 2,4,6,2′,4′,6′-hexanitrodiphenyl sulphide (HNDPS) and 2,4,2′,4′-tetranitrodiphenyl sulphide (TNDPS) using TLC.

Experimental

Compounds and solvents. The compounds were purified by passing them through neutral alumina columns, eluting with petroleum ether–ethyl acetate (95:5), and repeated crystallisations. They gave single spots in two-dimensional TLC and were spectroscopically pure; sorbic acid, m.p. 133–134°; vitamin A, m.p. 63–64°; α-carotene, m.p. 187–188°; β-carotene, m.p. 184°; γ-carotene, m.p. 178°, lycopene, m.p. 175°; and β-citraurin, m.p. 146–147°. Both 2,4,2′,4′-tetranitrodiphenyl sulphide, m.p. 196–197° and 2,4,6,2′,4′,6′-hexanitrodiphenyl sulphide, m.p. 234°, were prepared according to the procedure of BIELIG AND REIDIES[9]. All irrigating solvents were freshly dried and distilled.

Preparation, spotting and irrigation of plates. Photographic glass plates 35 × 12 cm were employed for the preparation of chromatoplates. A fine slurry of the adsorbent (50 g) in water (100 ml) was prepared. The slurry was poured on the glass plates and uniformly spread by tilting them from side to side. The plates were left at room temperature overnight for drying and activated at 110° in an oven for 1 h before use. The average coating of the adsorbents was noted after weighing the

TABLE I

R_F VALUES OF THE CHROMATOGRAMS RUN UNDER VARIOUS CONDITIONS

Serial No.	Compound	Silica Gel G		Silica Gel G–Kieselguhr G (3:1)		Neutral alumina–$CaSO_4$	
		Petroleum ether–benzene (3:2)	*Petroleum ether–methylene dichloride (9:1)*	*Petroleum ether–benzene (3:2)*	*Petroleum ether–methylene dichloride (9:1)*	*Benzene*	*Chloroform*
Plates not treated with nitro compounds							
1	Sorbic acid	0.24	0.15	0.28	0.19	0.15	0.31
2	Vitamin A	0.30	0.20	0.33	0.24	0.18	0.38
3	β-Citraurin	0.53	0.47	0.58	0.52	0.49	0.66
4	Lycopene	0.67	0.52	0.72	0.57	0.66	0.81
5	γ-Carotene	0.80	0.70	0.83	0.75	0.79	0.92
6	β-Carotene	0.87	0.87	0.92	0.92	0.88	0.97
7	α-Carotene	0.89	0.91	0.92	0.96	0.89	0.97
Plates treated with 0.1 % hexanitrodiphenyl sulphide							
1	Sorbic acid	0.06	0.01	0.11	0.08	0.02	0.12
2	Vitamin A	0.15	0.10	0.20	0.18	0.06	0.20
3	β-Citraurin	0.39	0.38	0.43	0.42	0.33	0.48
4	Lycopene	0.59	0.45	0.59	0.49	0.49	0.63
5	γ-Carotene	0.63	0.61	0.66	0.66	0.60	0.72
6	β-Carotene	0.68	0.73	0.72	0.78	0.69	0.78
7	α-Carotene	0.74	0.88	0.77	0.88	0.78	0.84
Plates treated with 0.1 % tetranitrodiphenyl sulphide							
1	Sorbic acid	0.16	0.07	0.21	0.14	0.09	0.22
2	Vitamin A	0.25	0.17	0.30	0.22	0.12	0.30
3	β-Citraurin	0.49	0.45	0.53	0.50	0.43	0.58
4	Lycopene	0.65	0.50	0.69	0.55	0.59	0.78
5	γ-Carotene	0.73	0.68	0.76	0.73	0.70	0.82
6	β-Carotene	0.78	0.80	0.82	0.85	0.79	0.88
7	α-Carotene	0.84	0.90	0.87	0.95	0.86	0.94

respective plates. The adsorbent coatings in various cases were: Silica Gel G, 5.12 mg/cm²; Silica Gel G–Kieselguhr G (3:1), 6.88 mg/cm²; neutral alumina (M. Woelm) containing 20 % $CaSO_4$, 7.2 mg/cm². The plates were impregnated with the respective nitro compounds by irrigating them with a 0.1 % solution in acetone using an ascending technique. The plates were dried at room temperature and spotted with 2 μg of each poly-unsaturated compound (dissolved in 5 μl of ethanol) with a standard micro-capillary. At the same time, these compounds were spotted on untreated chromatoplates. Both treated and untreated plates, after spotting, were irrigated at 28 ± 1° with suitable solvents employing an ascending technique. The irrigating solvent for the treated plates also contained 0.1 % of the nitro compound. All the compounds were located by spraying the chromatoplates with 0.5 % potassium permanganate

in 50 % sulphuric acid. The compounds could be seen as white spots against a brown background and were marked.

Fig. 1 is a typical chromatoplate showing the resolution of π complexes of conjugated compounds with TNDPS. The R_F values are given in Table I.

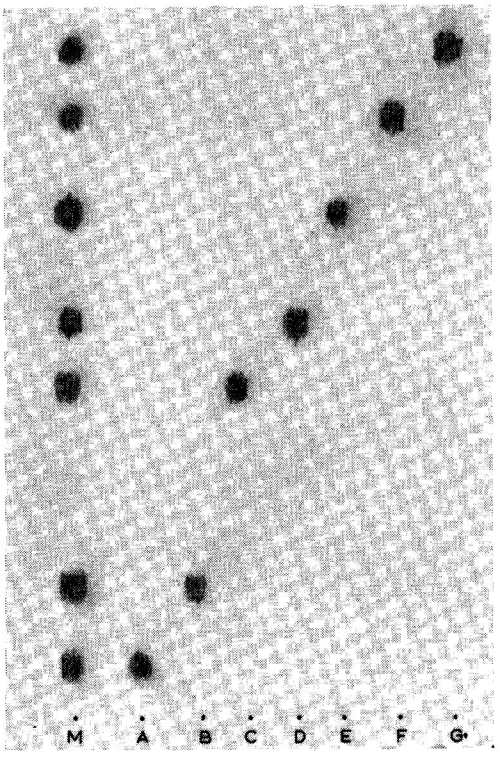


Fig. 1. Separation of conjugated compounds on a thin-layer chromatogram employing Silica Gel G, impregnated with 2,4,2',4'-tetranitrodiphenyl sulphide. M = mixture; A = sorbic acid; B = vitamin A; C = β-citraurin; D = lycopene; E = γ-carotene; F = β-carotene; G = α-carotene. Solvent, petroleum ether–methylene dichloride (9:1) containing 0.1 % of the nitro compound. Technique, ascending.

Discussion and results

It was interesting to observe that conjugated compounds like sorbic acid, vitamin A, α-, β-, and γ-carotene, lycopene and β-citraurin being π donors have better resolutions as complexes with π acceptors such as TNDPS and HNDPS. The spots of the complexes were quite distinct compared to when they were run as the uncomplexed materials. In general the R_F values of the complexes were lower than for the respective unsaturated compounds. The distance by which α- and β-carotenes are separated is far greater when they move as complexes than in the uncomplexed state. In addition to their conjugated nature, the presence of an α-ionone ring in α-carotene could be responsible for the stronger charge transfer and hence better resolution. Substitution of open chain in place of α- and β-ionone groups very much alters the migration of the complexes. The π complexes with γ-carotene have higher migration

than those with lycopene. Again the number of conjugated double bonds plays a specific role. The differences in the mobilities of complexes with HNDPS and TNDPS are due to the enhanced π accepting properties of the first compound compared with the latter.

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1 M. J. S. DEWAR, *Bull. Soc. Chim. (France)*, 18 (1951) 79; *Sci. Prog.*, 40 (1952) 604.
2 V. MASSEY AND H. GANTHER, *Biochemistry*, 4 (1965) 1161.
3 N. J. PALLERONI AND M. J. R. DE PRITZ, *Nature*, 185 (1960) 688.
4 L. M. DZIALOSZYNSKI, E. M. MYSTKOWSKI AND C. P. STEWART, *Biochem. J.*, 39 (1945) 63.
5 N. I. KRINSKY, D. G. CORNWELL AND J. L. ONCLEY, *Fed. Proc.*, 15 (1956) 113.
6 N. I. KRINSKY, D. G. CORNWELL AND J. L. ONCLEY, *Arch. Biochem. Biophys.*, 73 (1958) 233.
7 S. KRISHNAMURTHY, S. MAHADEVAN AND J. GANGULY, *J. Biol. Chem.*, 233 (1958) 32.
8 C. P. GARBER, J. GILLMAN AND M. REISACH, *Biochem. J.*, 75 (1960) 124.
9 H. J. BIELIG AND O. REIDIES, *Chem. Ber.*, 89 (1956) 580.

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CHROM. 5391

Separation of isomeric thioureas, thiazoles and thiazolines by thin-layer chromatography

During investigations of the condensation of unsaturated[1] and saturated[2] ketones on monoaryl and *sym.*-diaryl thioureas[3] in the presence of bromine or iodine, it was necessary to study the separation and identification of the final products and the optimum conditions for their maximum yield. Thin-layer chromatography (TLC), which has shown wide applicability in recent years, has advantages in simplicity and unsophisticated instrumentation and in particular in its quickness and better separation compared with paper chromatography, and suggests itself as a suitable technique for this purpose. During the investigations, it was evident that it is not only possible to separate thiazoles from the corresponding thioureas, but also to separate isomeric thioureas, thiazoles and thiazolines from one another. A large number of solvent systems[4] were tried, out of which six solvent systems as in Table I gave promising results.

An equimolecular mixture of: (a) *o*- and *p*-carboxyphenylthiourea, (b) *sym.*-di-*o*-carboxyphenylthiourea and *sym.*-di-*p*-carboxyphenylthiourea, (c) 2-*o*-carboxyphenylamino-4-methylthiazole and 2-*p*-carboxyphenylamino-4-methylthiazole, and (d) 2-*m*-chlorophenylimino-3-*m*-chlorophenyl-4-methyl-Δ^4-thiazoline and 2-*p*-chlorophenylimino-3-*p*-chlorophenyl-4-methyl-Δ^4-thiazoline, were successfully separated from one another on TLC plates in each of the six solvent systems.

Experimental

The TLC applicator used is an adjustable applicator Model S II and the adsorbent used for this technique was Silica Gel G for TLC with binding material. The well cleansed glass plates were coated with the adsorbent to a thickness of 250 μ

TABLE I

R_F VALUES OF THE VARIOUS COMPOUNDS IN VARIOUS SOLVENT SYSTEMS

Solvents: A = benzene–methanol (50:50); B = benzene–absolute ethanol (50:50); C = benzene–*n*-propanol (50:50) D = benzene–isopropanol (50:50); E = benzene–*n*-butanol (50:50); F = *n*-butanol–isopropanol (50:50).

Compound	*R_F values in solvent systems*					
	A	*B*	*C*	*D*	*E*	*F*
o-Carboxyphenylthiourea	0.85	0.75	0.87	0.72	0.85	0.77
p-Carboxyphenylthiourea	0.40	0.234	0.14	0.128	0.36	0.41
sym.-Di-*o*-carboxyphenylthiourea	0.88	0.87	0.85	0.82	0.81	0.78
sym.-Di-*p*-carboxyphenylthiourea	0.39	0.36	0.34	0.32	0.39	0.38
2-*o*-Carboxyphenylamino-4-methylthiazole	0.79	0.73	0.78	0.76	0.75	0.73
2-*p*-Carboxyphenylamino-4-methylthiazole	0.48	0.46	0.42	0.48	0.51	0.55
2-*m*-Chlorophenylimino-3-*m*-chlorophenyl-4-methyl-Δ^4-thiazoline	0.62	0.58	0.61	0.56	0.60	0.59
2-*p*-Chlorophenylimino-3-*p*-chlorophenyl-4-methyl-Δ^4-thiazoline	0.94	0.91	0.95	0.85	0.92	0.88
Time of development (min)	35	45	40	42	45	40

and the developing time was 35–45 min, differing from solvent to solvent. After the development was over, in the case of the monoaryl thioureas, the spots were visualised by spraying the plates with Feigl's reagent[5] (0.05 *N* iodine in 50 % ethanol containing 1.5 % sodium azide) followed by a starch solution spray, when colourless spots on a bluish background were obtained. In the case of the *sym.*-diaryl thioureas, the visualising agent used was Tollens' reagent when grey spots against a colourless background were observed. In the case of thiazoles, 5 % Na_2CO_3 solution followed by freshly prepared diazotised sulphanilic acid solution was used as the visualising agent when orange-red coloured spots on a whitish background were obtained. In the case of the thiazolines, a 0.5 % solution of acidified $KMnO_4$ was used as the visualising agent when white spots against a pink background were obtained. In all the above cases the spots were compact, and well separated from one another.

The details of the R_F values in the various solvent systems and the times of development are given in the Table I.

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1 H. TRIPATHY, B. C. DASH AND G. N. MAHAPATRA, *Indian J. Chem.*, 8 (1970) 586.
2 B. C. DASH AND G. N. MAHAPATRA, *Indian J. Chem.*, 5 (1967) 40.
3 G. N. MAHAPATRA AND B. C. DASH, *J. Proc. Inst. Chem.*, 39 (1967) 178.
4 G. GURU, *Thesis*, Utkal University, Bhubaneswar, India, 1970.
5 B. K. PATNAIK, B. C. DASH, D. P. DAS AND G. N. MAHAPATRA, *Curr. Sci.*, 23 (1966) 595.

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CHROM. 5401

Separation of indole derivatives and catecholamines by thin-layer chromatography

Various papers have been published concerning thin-layer chromatographic (TLC) separation of tyrosine and tryptophan metabolites from norepinephrine (NE)[1–5] and serotonin[7–12]. GIESE *et al.*[5] succeeded in separating NE and its metabolites, CHOULIS[2] successfully separated dopamine, epinephrine and NE. SCHNEIDER AND GILLIS[10] achieved a satisfactory separation of dopamine, DOPA and NE by a two-dimensional run. SANDLER AND RUTHVEN[8] reported R_F values for catecholamines (CA) found with different solvent systems. COWLES *et al.*[3], AURES *et al.*[1] and PILLAY AND MEHDI[7] described the separation of indole compounds.

All the R_F values determined by the above authors were unsuitable, or alkali solutions had been used which contributed to the decomposition of the substances in question.

For a series of pharmacological and enzymic kinetic experiments, it appears desirable, however, to separate all NE and 5-HT precursors and metabolites by TLC. Recently FLEMING AND CLARK[4] reported a method which permits isolation and identification of CA by TLC. The required two-dimensional run in their method, however, is time-consuming and has additional difficulties connected with the evaluation of radioisotope experiments.

In this paper, a method is reported for the TLC separation of tyrosine, DOPA, Dopamine and NE, as well as the main metabolites normetanephrine and 3-methoxy-4-hydroxymandelic acid by a one-dimensional run. Isolation, identification and quantitative measurement of the tryptophan metabolites (serotonin route) is possible using the same technique.

For these substances, the solvent system of GIESE *et al.*[5] proved to be successful.

Experimental

Materials. 1, L-tyrosine, puriss; 2, L-3-(3,4-dihydroxyphenyl)alanine (DOPA), puriss; 3, 3-hydroxytyramine hydrochloride (dopamine); 4, L-norepinephrine hydrochloride; 5, D,L-normetanephrine hydrochloride; 6, D,L-4-hydroxy-3-methoxymandelic acid; 7, D,L-5-hydroxytryptophan (5-HTP); 8, D,L-tryptophan; 9, 5-hydroxytryptamine (serotonin); 10, 5-hydroxyindoleacetic acid (5-HIAA); 11, urea; 12, tryptamine; 13, dimethyltryptamine (DMT); 14, bufotenine; 15, 5-methoxyindoleacetic acid (5-MeOIAA); 16, indoleacetic acid (IAA); 17, melatonine. Suppliers were Fluka (1–3, 6–8, 10–17), Roth (5, 9), Merck (11) and Hoechst (4).

Solvent systems. (A) *n*-Butanol–5 *N* acetic acid (100:35). (B) Butan-2-one (ethyl methyl ketone)–acetone–2.5 *N* acetic acid (40:20:20).

Detection. The spots of the CA were visualized by spraying the plates with *p*-nitroaniline[13]. The indoles were sprayed with *p*-dimethyl aminocinnamic aldehyde (*p*-DMCA).

Thin-layer plates. For the indoles, using cellulose MN 300 (Macherey & Nagel, Düren, G.F.R.), plates were prepared by mixing 16 g cellulose and 100 ml water with an ESGE stirrer. Plates were coated by means of a Camag model to a thickness of

0.3 mm and were dried in the open air. For the CA, however, precoated cellulose plates, 20 × 20 cm, (Schleicher & Schüll, Dassel, G.F.R.) gave better results.

Separation of compounds. The tyrosine metabolites were dissolved in water at a temperature of 22°. No change in chromatographic behaviour could be found by attempting to dissolve all substances in 0.01 *N* HCl. Indoles were dissolved in a solution containing dilute HCl and methanol.

For the indoles, 50 ng in 10 μl were always applied, and for the CA, 1 μg in 10 μl.

The chambers with saturated solutions were protected against the influence of direct light. Chromatograms were ascending, one-dimensional.

Quantitative analysis of the indoles. Quantitative analysis was by remission or transmission in a Zeiss spectrophotometer for TLC (No. CA 2).

TABLE I

TLC BEHAVIOUR OF TYROSINE DERIVATIVES TOWARDS NE ON CELLULOSE-PREPARED PLATES (SOLVENT SYSTEM B)

Running time for one running procedure; 2–2 ½ h.

Substance	*hR_F values*	*Colour impression*
Tyrosine	29	Pink
DOPA	16	Blue–grey
Dopamine	52	Pale blue
Norepinephrine	38	Grey–greyish blue
Normetanephrine	47	Blue–lilac
3-Methoxy-4-hydroxymandelic acid	89	Lilac

Results and discussion

Table I shows the separation of the tyrosine derivatives on cellulose-precoated plates, from which it can be seen that the sharpness of separation of these compounds was excellent. The method was applicable to both mixed and pure substances. Variations in the distance of travel from the origin showed that a sharp separation could also be attained by shorter distances (12 cm; compared with the usual 18.5 cm).

Table II shows the separation of some tryptophan metabolites. For the examination of 5-HTP metabolism in the brain, we used solvent system A, which is suitable for the separation of radioactively labelled 5-HTP, 5-HT and 5-HIAA (unpublished results).

500 ng is quoted[11] as the lowest detection limit, when serotonin is stained with *p*-DMCA on silica gel. Polyamide plates are not suitable for indole visualization with *p*-DMCA because of the green staining on the layer[6]. Using the present method, 5 ng of serotonin can be detected using cellulose as sorbent. Quantitative densitometric analysis of serotonin is possible by transmission measurement down to 10 ng (to be published). This technique is not applicable to animal experiments, where urea and tryptophan are co-chromatographed, since their R_F values are unsuitable for the densitometric determination of serotonin.

A solvent system therefore had to be found for the determination of serotonin in tissue which satisfactorily separated the physiological substances 1–5 (Table II). Of the sixty solvent systems, which were tested on small-scale chromatograms

TABLE II

TLC SEPARATION OF TRYPTOPHAN METABOLITES OF THE INDOLE TYPE ON CELLULOSE PLATES (SOLVENT SYSTEMS A AND B)

The running time of the solvent system A is 2 h and of processing solution B it is 1 h, with a separation run of 12 cm.

No.	*Substance*	hR_F *values*		*Colour impression*[a]
		A	*B*	
1	D,L-5-HTP	15	35	Blue
2	D,L-Tryptophan	33	50	Lilac
3	Serotonin	35	65	Blue
4	5-HIAA	85	98	Blue
5	Urea	40	57	Red
6	Tryptamine	71	65	Lilac
7	DMT	72	71	Lilac
8	Bufotenin	47	75	Blue
9	5-MeOIAA	98	98	Blue
10	IAA	98	98	Lilac
11	Melatonin	98	98	Blue

[a] It is observed in the colour reaction that the replacement of HCl in position 5 in the indole ring by a hydroxy or methoxy group causes a change of colour from violet to blue.

(3 × 10 cm), it was found that alkaline solvent systems could not be used because of the sensitivity of the substances. Use of the alcohol- and ester-containing solvent systems had to be restricted, as serotonin formed a widely scattered spot. The combination of water, acetic acid and acetone yielded hR_F values around 98. We therefore used a mixture of a higher-molecular-weight ketone — ethyl methyl ketone — in which the solubility of the substances was reduced.

Solvent system B, obtained in this manner, is especially suitable for the separation of the substances 1–5 (Table II) and allows densitometric determination of serotonin and tryptophan. 5-HIAA is not suitable for densitometric determinations because of high R_F values, whereas 5-HTP is not suitable because of the widely scattered spotting. Experiments using this method in animal tissue, especially brain, are in progress.

The TLC separation of the NE precursors and main metabolites achieved may be a good method for some enzymic kinetic examinations of NE formation. The quantitative estimation of radioactive compounds on a single plate with a one-dimensional run seems possible.

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1 D. AURES, R. FLEMING AND R. HÅKANSON, *J. Chromatogr.*, 33 (1968) 480.
2 N. H. CHOULIS, *J. Pharm. Sci.*, 56 (1967) 196.
3 E. J. COWLES, G. M. CHRISTENSEN AND A. C. HILDING, *J. Chromatogr.*, 35 (1968) 389.
4 R. M. FLEMING AND W. G. CLARK, *J. Chromatogr.*, 52 (1970) 305.
5 J. GIESE, E. RÜTHER AND N. MATUSSEK, *Life Sci.*, 6 (1967) 1975.

6 J. HARLEY-MASON AND A. A. P. G. ARCHER, *Biochem. J.*, 69 (1958) 60P.
7 D. T. N. PILLAY AND R. MEHDI, *J. Chromatogr.*, 32 (1968) 592.
8 M. SANDLER AND C. R. J. RUTHVEN, in I. SMITH (Editor), *Chromatographic Electrophoretic Techniques, Vol. I*, 3rd ed., W. Heineman Ltd., London, pp. 714–746.
9 E. SCHMID, *Arch. Kreisl. Forsch.*, 49 (1966) 83.
10 H. G. SCHNEIDER AND C. N. GILLIS, *Biochem. Pharmacol.*, 14 (1965) 623.
11 E. STAHL (Editor), *Dünnschichtchromatographie*, Springer, Berlin, New York, 1967.
12 H. STROBACH, *Z. Klin. Chem. Klin. Biochem.*, 5 (1967) 77.
13 W. VON STUDTNITZ, *Scand. J. Clin. Lab. Invest.*, 12 (1960) suppl. 48.

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CHROM. 5420

Identification simple de treize additifs dans les aliments composés par chromatographie sur couche mince

L'alimentation animale moderne fait appel à un nombre de plus en plus important d'additifs. Ceci constitue un grand problème pour l'analyste, qu'il appartienne aux laboratoires de contrôle de fabrication des aliments, ou aux Services officiels (Répression des Fraudes). Les doses incorporées sont de plus en plus faibles, et l'aliment constitue un milieu très complexe. Des méthodes d'analyse quantitative existent pour chacun des additifs, mais il n'est pas concevable de les appliquer successivement et systématiquement au contrôle de l'étiquetage, à la recherche de l'erreur de fabrication ou de la fraude éventuelle. Seule une détection préalable rapide du ou des additifs présents, peut rendre possible un contrôle efficace et généralisé. La méthode que nous proposons répond à cet objectif, en permettant d'identifier treize substances (Tableau I) parmi celles autorisées en France, et particulièrement les plus récemment admises.

Une première identification du zoalène, de la nitrofurazone, de la furazolidone et de l'amprolium a été proposée[1], utilisant la chromatographie sur couche mince; la présence de pigments (luzerne) rend la détection difficile. Dix-huit médicaments utilisés dans les aliments médicamenteux et la supplémentation, ont pu être identifiés à l'état pur, également par chromatographie sur couche mince[2]. Enfin, des auteurs anglais[3] ont étendu la première méthode[1] à la détection de dix-sept additifs dans les

TABLEAU I

LISTE DES ADDITIFS IDENTIFIÉS PAR LA MÉTHODE PROPOSÉE

Nom commercial	*Doses autorisées en France en p.p.m.*	*Dénomination chimique*
Furazolidone	25	3-(5-Nitrofurfurylidèneamino)-2-oxazolidinone
Nitrofurazone	50	5-Nitrofurfuraldéhyde semicarbazone
Payzone (Nitrovin)	12	Chlorhydrate de 1,5-bis(5-nitro-2-furyl)penta-1,4-diène-3-one-amidinohydrazone
Zoalène (D.O.T.)	75	3,5 Dinitro-*o*-toluamide
Dimétridazole	150	1,2 Diméthyl-5-nitroimidazole
Ronidazole	60	(1-Méthyl-5-nitroimidazol-2-yl) méthylcarbamate
Coyden (clopidol)	125	3,5-Dichloro-2,6-diméthyl-4-pyridinol
Buquinolate	80	4-Hydroxy-6,7-di-isobutoxy-3-quinolinecarboxylate d'éthyle
Décoquinate (Deccox)	10	Éthyl 6-décycloxy-7-éthoxy-4-hydroxyquinoline-3-carboxylate
Méthylbenzoquate (Statyl)	10	Méthyl 7-benzyloxy-6-butyl-1,4-dihydroxyquinoléine-3-carboxylate
Carbadox (GS 6244)	(50)[a]	3-Méthyl-(2-quinoxalinylméthylène) carbazate N^1,N^4 dioxyde
Ethopabate	8	Méthyl-4-acétamido-2-éthoxybenzoate
Amprolium	125	Chlorhydrate de chlorure de 1-(4-amino-2-*n*-propyl-5 pyrimidinylméthyl)-2-picolinium

[a] Autorisé aux U.S.A.

aliments composés, avec un nombre limité de systèmes de solvants et de révélateurs. Nous avons entrepris simultanément un travail identique portant sur l'identification de treize additifs, dont un certain nombre récemment admis dans les aliments composés. Étant donné le nombre plus restreint de substances envisagées, nous avons abouti à une méthode plus simple, supprimant la chromatographie préalable sur colonne d'alumine[3], et pouvant s'appliquer ainsi à des séries d'échantillons. Elle consiste essentiellement dans l'extraction successive d'une même prise d'essai de l'aliment par quatre solvants (hexane, acétone, chloroforme et méthanol), le premier permettant d'éliminer un certain nombre de substances interférentes. Les chromatographies sur couche mince des extraits sont faites sur deux supports différents (silice fluorescente et alumine fluorescente) et comportent deux systèmes de solvants d'élution et deux types de révélateurs. Cette méthode permet de détecter les doses autorisées de chacun des additifs (Tableau I), et pour bon nombre d'entre eux le cinquième de cette dose (10 à 20 p.p.m.).

Méthode

Réactifs. Tous les réactifs sont de qualité "pour analyse": Hexane, acétone, chloroforme, méthanol, méthanol ammoniacal (2.5 %), acétate d'éthyle, chlorure de méthylène, ether éthylique, Gel de Silice GF_{254} (Merck), Alumine GF_{254} (Merck).

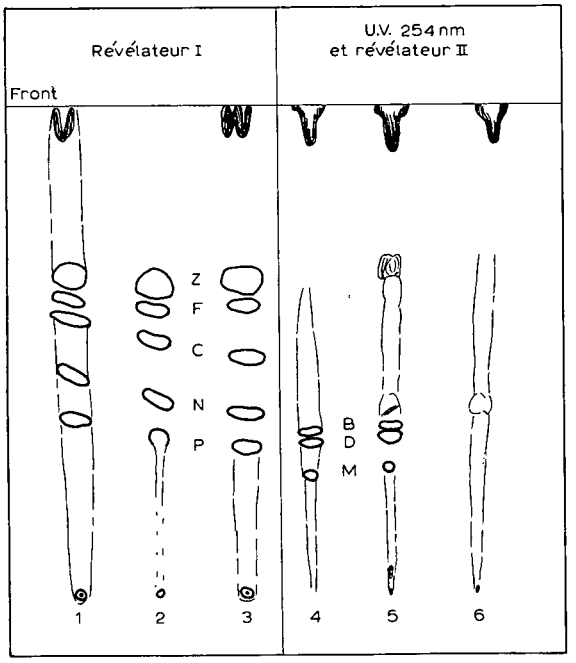


Fig. 1. (1) Extrait 1 d'aliment témoin, surchargé d'additifs purs; (2) additifs purs; (3) extrait 1 d'aliment; (4) extrait 2 d'aliment témoin, surchargé d'additifs purs; (5) extrait 2 d'aliment; (6) extrait 2 d'aliment témoin. P = Payzone, rouge brun; N = nitrofurazone, rouge brun; C = carbadox, jaune orangé; F = furazolidone, rouge brun; Z = zoalène, violet; M = méthylbenzoquate; D = dimétridazole; B = buquinolate, M, D et B sont rouge brun après révélation II, et violet foncé sous UV.

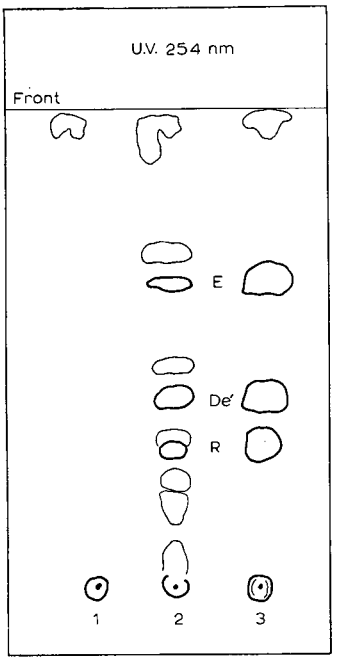
Systèmes de développement: (A) acétate d'éthyle–chlorure de méthylène (1:1), (B) chloroforme–méthanol (9:1).

Révélateurs (I) éthylène diamine, (II) réactif de Dragendorff modifié[1]. Solution-stock: Dissoudre 8 g de sous-nitrate de bismuth dans 5 ml d'acide nitrique pur, ajouter 15 ml d'eau. Verser cette solution dans une suspension de 20 g d'iodure de potassium dans 1 ml d'acide chlorhydrique 6 *N* et 5 ml d'eau. Compléter à 100 ml avec de l'eau (cette solution se conserve plusieurs mois). Solution pour la pulvérisation: À 5 ml de solution stock, ajouter 5 ml d'acide chlorhydrique concentré et 20 ml d'eau (cette solution se conserve au moins 10 jours).

Appareillage. Matériel classique pour la chromatographie sur couche mince. Les plaques (20 × 20 cm) d'épaisseur 0.25 mm, sont confectionnées suivant la technique habituelle, et activées à 110° durant 1 h avant emploi.

Extraction des additifs. Peser 10 g d'aliment en farine dans un tube à centrifuger de 100 ml, ajouter 50 ml d'hexane, boucher et agiter fortement durant 5 min, centrifuger quelques minutes, et éliminer le surnageant par pipettage. Répéter une fois l'opération.

Ajouter 40 ml d'acétone au culot d'aliment, agiter fortement 5 min, centrifuger quelques minutes, et prélever le surnageant dans un ballon à évaporer de 250 ml. Répéter une fois l'opération. Réduire sous vide les extraits acétoniques réunis jusqu'à



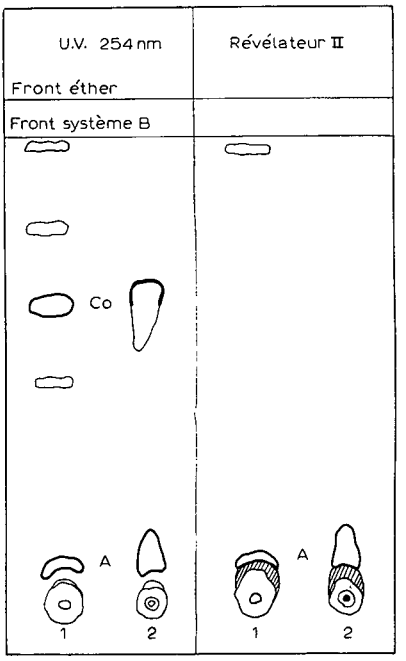


Fig. 2. (1) Extrait 1 d'aliment témoin; (2) extrait 1 d'aliment; (3) extrait 1 d'aliment témoin surchargé d'additifs purs. R = Ronidazole; Dé = décoquinate; E = ethopabate, R, Dé et E sont violet foncé sous UV et rouge brun après révélation II pour les surcharges.

Fig. 3. (1) Extrait 3 d'aliment; (2) extrait 3 d'aliment témoin, surchargé d'additifs purs. A = Amprolium, violet foncé sous UV, rouge après révélation II; Co = coyden, violet foncé sous UV.

1 à 2 ml, transvaser dans un petit récipient et protéger de la lumière: ceci constitue l'extrait 1.

Ajouter 40 ml de chloroforme au culot d'aliment, agiter fortement durant 5 min, et verser rapidement sur un filtre plissé placé dans un entonnoir au-dessus d'un ballon à évaporer de 100 ml. Laisser filtrer complètement, conserver l'aliment dans le filtre, évaporer l'extrait chloroformique jusqu'à 1 à 2 ml, transvaser dans un petit récipient: ceci constitue l'extrait 2.

Remettre l'aliment retenu sur le filtre dans le tube à centrifuger, ajouter 40 ml de méthanol ammoniacal, agiter fortement durant 5 min, centrifuger. Prélever le surnageant dans un ballon à évaporer de 250 ml. Evaporer jusqu'à 1 à 2 ml, transvaser dans un petit récipient: ceci constitue l'extrait 3.

Séparation et identification

(*a*) *Chromatographie sur Silice GF_{254} fluorescente*

1. Déposer sur une moitié de plaque des taches de 20 à 50 μl d'un ou plusieurs extraits 1, encadrées de taches témoin du mélange: payzone, nitrofurazone, furazolidone, zoalène et carbadox. Déposer sur la seconde moitié des taches de 20 à 50 μl d'un ou plusieurs extraits 2, encadrées de taches témoin du mélange: méthylbenzoquate, dimétridazole et buquinolate. Développer par le système A sur 10 cm environ, retirer la plaque, sécher à l'air chaud, puis développer par le système B sur 15 cm. Retirer la plaque, sécher sous air chaud. Révéler la moitié de plaque correspondant à l'extrait 1 en pulvérisant le révélateur I et en protégeant l'autre partie. Observer sous lumière ultra-violette (254 nm) la seconde moitié, puis la révéler par pulvérisation du révélateur II. Interpréter chaque étape à l'aide de la Figure 1.

2. Déposer des taches de 20 à 50 μl d'un ou plusieurs extraits 1, encadrées par des taches témoin du mélange: éthopabate, décoquinate et ronidazole. Développer par le système A sur 15 cm, retirer la plaque, sécher sous air chaud, observer sous lumière ultra-violette (254 nm), puis révéler par pulvérisation du révélateur II. Interpréter à l'aide de la Figure 2.

(*b*) *Chromatographie sur Alumine GF_{254} fluorescente*

Déposer des taches de 20 à 50 μl d'extrait 3, encadrées par des taches témoin du mélange: coyden et amprolium. Faire une préélution dans l'éther sur 15 cm, retirer la plaque, sécher, puis développer par le système B sur 15 cm. Observer sous lumière ultra-violette (254 nm), puis révéler par pulvérisation du réactif II. Interpréter à l'aide de la Figure 3.

Discussion

Cette méthode permet d'identifier les substances aux doses autorisées (Tableau I), mais pour certaines d'entre elles (zoalène, payzone, nitrofurazone, amprolium), la limite de détection est de l'ordre du cinquième de cette dose. Pour les additifs présents aux plus faibles doses (décoquinate, ethopabate, méthylbenzoquate) et particulièrement lors de l'examen sous UV, où de nombreuses taches sont visibles, un doute peut exister. Il est alors nécessaire de répéter une nouvelle chromatographie en encadrant chaque extrait, d'une part par le mélange des additifs purs, d'autre part par l'extrait surchargé avec le même mélange. Ceci peut être également utile lorsque les extraits sont chargés de substances interférentes qui peuvent modifier la migration et la qualité de la séparation. La chromatographie simultanée du coyden et de l'am-

prolium (système B) peut conduire, suivant la nature et l'importance des substances interférentes, à une interprétation difficile pour l'amprolium, son R_F étant faible; le doute peut être levé complètement en effectuant une migration complémentaire dans le méthanol pur.

Nous ne donnons aucun R_F indicatif, mais seulement l'ordre de séparation, la référence aux mélanges de substances pures ou aux surcharges étant beaucoup plus utile, et pouvant éviter des erreurs d'interprétation.

Les extractions et les chromatographies peuvent être réalisées en série, permettant le traitement simultané de plusieurs échantillons d'aliment.

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1 H. Z. KNAPSTEIN, *Z. Anal. Chem.*, 217 (1966) 181.
2 J. J. ANTKOWIAK AND A. L. SPATORICO, *J. Chromatogr.*, 29 (1967) 277.
3 P. W. HAMMOND AND R. E. WESTON, *Analyst (London)*, 94 (1969) 921.

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* Avec la collaboration technique de EVELYNE BIETTE.

CHROM. 5405

Identification des sucres contenus dans un extrait végétal et évaluation de leurs teneurs individuelles par chromatographie et photodensitometrie

Depuis le célèbre travail de PARTRIDGE[1], la séparation et l'identification des sucres par chromatographie a reçu une amélioration grâce à la chromatographie en couche mince.

Les supports suivants ont été utilisés: polyamide[2], gel de silice[3–5], polycarbonate[6], cellulose[7–9]. La poudre de cellulose à permis la meilleure séparation des sucres de nos extraits.

Pour l'évaluation quantitative, la photodensitométrie employée depuis 1950 (bibl. 10, 11) et tout récemment par DE STEFANIS ET PONTE[3] nous a paru être le procédé le plus commode.

Séparation chromatographique

Mode opératoire

Il est voisin de celui de notre précédent travail[12] et n'en diffère que par les points suivants.

Développement. On utilise la phase supérieure de mélange butanol–acide acétique–eau (4:1:5) préparé depuis plus de trois semaines et moins de trois mois. Afin d'avoir un étalement suffisant des R_F, on procède à une deuxième migration dans le même sens après avoir laissé sécher la plaque horizontale toute la nuit.

Révélation. Le composé formé par l'acide tartrique et l'aniline a un coefficient d'extinction moléculaire supérieur à celui formé avec les autres acides organiques, y compris l'acide phtalique ou l'acide phosphorique. Le révélateur retenu pour les aldoses est le mélange: acide tartrique (1.50 g)–aniline (0.93 ml)–butanol saturé d'eau (100.00 ml).

On chauffe pendant 10 min à 100°; les aldoses donnent des taches marron.

Pour les autres sucres, nous avons retenu le mélange: aldéhyde anisique (5 ml) mélangé extemporanément à: alcool éthylique (90 ml)–acide sulfurique $d = 1.8$ (5 ml)–acide acétique (1 ml).

Par chauffage à 100°, pendant 4 min environ, on obtient des taches roses. L'évolution de la coloration est observée à travers un hublot. L'homogénéité du fond est améliorée par rotation constante de la plaque à l'intérieur de l'étuve (Fig. 1).

Les résultats sont reproduits sur la Fig. 2.

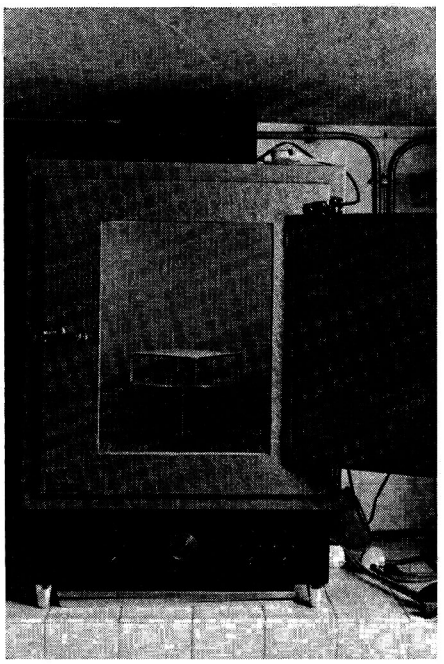
Les R_F des différents sucres étudiés et leur R_F' (après double migration) sont groupés dans le Tableau I. (Le solvant de migration utilisé était préparé depuis deux mois.)

Discussion

L'âge du solvant a une incidence importante sur la netteté de la séparation et sur la valeur des R_F. Les développements réalisés avec un solvant préparé depuis moins de trois semaines, présentent des trainées; avec un solvant de plus de trois mois il y a diminution des R_F.

Les R_F de sucres autres que ceux contenus dans nos extraits sont groupés dans le Tableau II.

Ces sucres n'interfèrent ni avec les précédents ni entre eux.



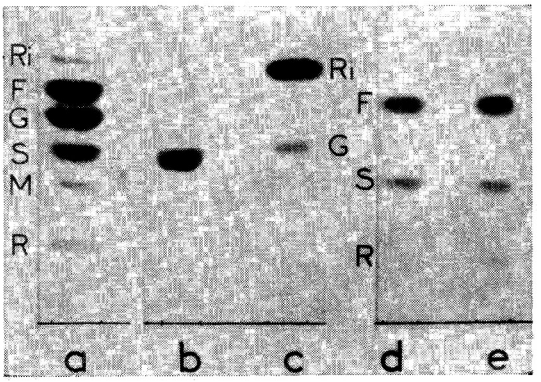


Fig. 1. Vue de l'étuve avec hublot et dispositif permettant la rotation de la plaque.

Fig. 2. (a) Autoradiographie de chromatogramme de sucres marqués par incorporation de $^{14}CO_2$. (b et d) extrait de feuille (*Vitis vinifera*). (c et e) solution témoin. (b et c) révélation tartrate d'aniline. (d et e) révélation aldehyde anisique. R = raffinose, M = maltose, S = saccharose, G = glucose, F = fructose, Ri = ribose.

TABLEAU I

VALEUR COMPARÉE DES R_F EN SIMPLE ET EN DOUBLE MIGRATION

	R_F	R_F'
Raffinose	0.14	0.26
Maltose	0.22	0.39
Saccharose	0.29	0.48
Glucose	0.33	0.55
Fructose	0.37	0.60
Ribose	0.43	0.68

Evaluation photodensitometrique

Mode opératoire. Les mesures sont effectuées dans les mêmes conditions que dans notre précédent travail[12].

L'alternance des R_F des aldoses et des autres sucres permet en employant les deux révélateurs sur deux plaques distinctes portant un même échantillon, d'augmenter l'intervalle entre deux taches successives; ainsi le scripteur du photodensitomètre peut regagner la ligne de base entre deux taches.

Un filtre Wratten 47B est placé entre la plaque chromatographique et la cellule

quand le tartrate d'aniline est employé comme révélateur; on n'utilise aucun filtre pour les plaques révélées par l'aldéhyde anisique.

TABLEAU II

R_F DE SUCRES ABSENTS DE NOS EXTRAITS VÉGÉTAUX ET R_F' OBTENU EN DOUBLE MIGRATION

	R_F	R_F'
Stachyose	0.05	0.07
Lactose	0.16	0.30
Mélibiose	0.24	0.42
Galactose	0.31	0.52
Mannose	0.35	0.58
Arabinose	0.36	0.59
Xylose	0.40	0.65

La surface des pics est déterminée par totalisation des "tops" inscrits par le photodensitomètre. Cependant si la teinte de fond de la couche mince n'est pas homogène, il faut déterminer la surface des pics par pesée après découpage.

Résultats et discussion. La Fig. 3 montre la relation existant entre la quantité de sucre déposée et la surface du pic enregistré.

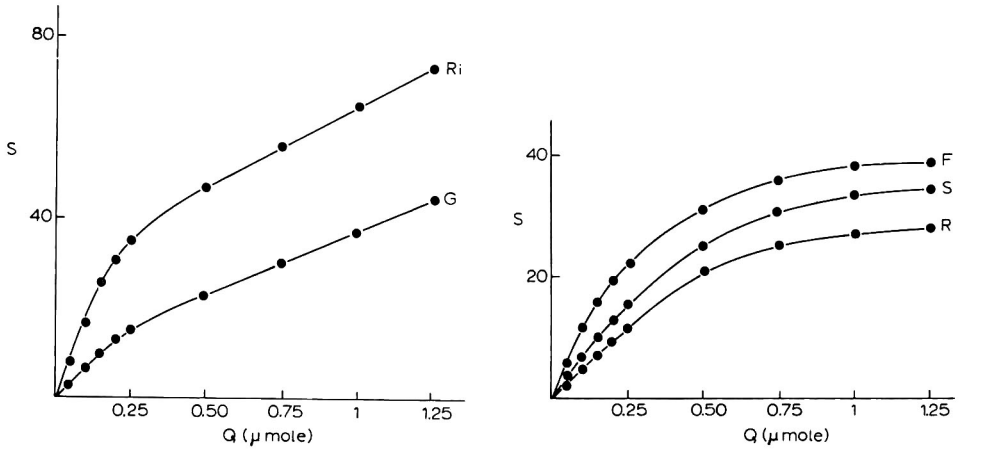


Fig. 3. Relation entre la surface d'un pic (déterminée par pesée exprimée en mg), et la quantité de sucre déposée exprimée en micromolécule gramme d'ose. Ri = Ribose, G = Glucose. Révélation par tartare d'aniline. R = Raffinose, S = Saccharose, F = Fructose. Révélation par aldehyde anisique.

Les dépôts doivent être compris entre 0.25 et 0.75 μmole d'ose. On détermine ensuite la valeur exacte de la quantité déposée par ajustement avec la courbe étalon au moyen d'un abaque.

La précision des résultats est de l'ordre de $\pm 8\%$. Comme dans toute micro-méthode, cette précision peut être grandement altérée par l'intervention subite d'une cause qui échappe au manipulateur (perte d'une partie de l'échantillon, irrégularité dans la révélation). Aussi est-il prudent de traiter chaque échantillon au moins deux fois sur des plaques différentes qui subiront séparément les diverses manipulations de la technique, soit quatre plaques par échantillon.

Les taches obtenues avec le tartrate d'aniline sont d'un contraste optimum 20 h après la révélation. Deux à trois jours après le contraste diminue du fait de l'apparition d'une teinte de fond.

Les taches obtenues avec l'aldéhyde anisique ont leur contraste optimum 3 à 5 h après la révélation. Elles s'estompent ensuite lentement.

Du fait de la présence de témoins ces évolutions de coloration ne portent pas atteinte à la précision de la méthode.

Conclusion

La technique chromatographique proposée permet d'obtenir, à partir d'un extrait végétal, la séparation selon des taches nettes des six sucres les plus courants de la biochimie végétale: ribose, fructose, glucose, saccharose, maltose, raffinose. Six autres sucres que nous n'avons pas rencontrés dans nos extraits peuvent également être identifiés sans interférer.

En révélant séparément les aldoses d'une part et les autres sucres d'autre part, on peut effectuer une évaluation photodensitométrique des teneurs individuelles avec une précision déjà acceptable.

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1 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
2 J. P. MARAIS, *J. Chromatogr.*, 27 (1967) 321.
3 V. A. DE STEFANIS ET J. G. PONTE, JR., *J. Chromatogr.*, 34 (1968) 116.
4 H. WIELE ET E. HORAK, *J. Chromatogr.*, 47 (1970) 527.
5 D. J. BELL ET M. Q.-K. TALUKDER, *J. Chromatogr.*, 49 (1970) 469.
6 M. BOUNIAS, *Chim. Anal.*, 51 (1969) 76.
7 A. SCHWEIGER, *J. Chromatogr.*, 9 (1962) 374.
8 M. L. WOLFROM, D. L. PATIN ET R. M. DE LEDERKREMER, *J. Chromatogr.*, 17 (1965) 488.
9 J. F. MC KELVY ET J. R. SCOCCA, *J. Chromatogr.*, 51 (1970) 316.
10 E. F. MC FARREN, K. BRAND ET H. R. RUTKOWSKI, *Anal. Chem.*, 23 (1951) 1146.
11 J. B. HIMES, L. D METCALFE ET H. RALSTON, *Anal. Chem.*, 33 (1961) 364.
12 M. BOURZEIX, J. GUITRAUD ET F. CHAMPAGNOL, *J. Chromatogr.*, 50 (1970) 83.

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* Avec la colloboration technique de Mme. H. COMPAN

CHROM. 5398

The paper chromatographic separation of naturally acetylated, formylated and pyruvylated oligosaccharides

The discovery of hydrolytic enzymes releasing pyruvylated, O-acetylated and O-formylated fragments from bacterial extracellular polysaccharides[1-3] led to a requirement for suitable systems for their chromatographic separation. Of particular importance was the separation of such fragments from the corresponding non-acylated material which may either be present naturally or as an artifact of preparation. The relatively large yields of oligosaccharides obtained by enzymic hydrolysis made paper chromatography a method of choice for preparative separation as well as for comparative studies.

Experimental

A number of naturally O-acetylated, O-formylated and pyruvylated oligosaccharides were available[1,2]. The complete carbohydrate structure of each was known, but the position of the acyl groups was only known for the pyruvylated fragments. The compounds tested and their known structures are shown in Table I.

TABLE I

FRAGMENTS ISOLATED FROM BACTERIAL EXOPOLYSACCHARIDES

Fraction	*Carbohydrate structure*	*Substituents*	[a]M_{Glca}	*Source*	*Refer*
F 1	Gal→Glc→GlcA→Fuc	—	0.46	*Escherichia coli* K27	1
F 2	Gal→Glc→GlcA→Fuc	Acetyl	0.41	*E. coli* K27	1
F 3	Glc→Glc→GlcA→Fuc	—	0.47	*Klebsiella aerogenes* Type 54	4
F 4	Glc→Glc→GlcA→Fuc	Formyl	0.47	*K.aerogenes* Type 54	2, 3
F 5	Glc→Glc→GlcA→Fuc	Formyl + acetyl	0.45	*K.aerogenes* Type 54	2, 3
F 6	Glc→GlcA→Fuc	Formyl	0.53	*K.aerogenes* Type 54	5
F 7	Glc→GlcA→Fuc	Formyl + acetyl	0.51	*K.aerogenes* Type 54	5
F 8	Gal→GlcA→Gal	—	0.54	*E.coli* K12	6
F 9	Gal→GlcA→Gal	Pyruvyl	0.87	*E.coli* K12	6
F 10	Gal	Pyruvyl	0.95	*E.coli* K12	6
F 11	Gal	Pyruvyl	0.95	*Salmonella typhimurium*	7

[a]Electrophoretic mobility relative to glucuronic acid in pyridinium acetate (pH 5.3).

The corresponding non-acylated fragments were obtained from the polysaccharides by partial acid hydrolysis, as were some of the pyruvylated fractions[4-6]. As the products of enzymic hydrolysis and also some of those obtained by acid hydrolysis contained varying amounts of salts these were removed by paper electrophoresis in pyridinium acetate buffer (pH 5.3). As well as oligosaccharides, two isomeric forms of pyruvylated galactose were tested, as such pyruvylated fragments are commonly found in bacterial exopolysaccharides. The first of these, the 4,6-carboxyethylidene derivative was obtained from the polysaccharide of *Escherichia coli* K12 by partial acid hydrolysis of the periodate–oxidised polymer[6], while the 3,4-carboxyethylidene galactose was prepared in the same way from the polysaccharide of a *Salmonella typhimurium* strain[7].

The solvents tested comprised a large number of those commonly employed for the paper chromatographic separation of sugars and oligosaccharides. Several of those tested were basic solvents, while others were acidic or neutral. From preliminary experiments it was clear that most of the solvents tested, failed to give satisfactory separation of the pairs of oligosaccharides which differed only in the acyl groups present. Four solvents were eventually selected for further study: (A) butan-1-ol–pyridine–water (6:4:3); (B) ethyl acetate–pyridine–acetic acid–water (5:5:1:3); (C) acetic acid–formic acid–ethyl acetate–water (3:1:18:4); and (D) butanol–acetic acid–water (4:1:5). All were run as descending systems at 20° using Whatman No. 1 paper. Irrigation times were normally 24 h for solvents A, B and C, and 96 h for solvent D. The carboxyethylidene sugars were run for 10 h, in solvent C. The oligosaccharides were applied as approximately 10 nmoles amounts and were detected after irrigation, with alkaline silver nitrate reagent.

TABLE II

THE PAPER CHROMATOGRAPHIC MOBILITIES OF ACYLATED AND OTHER OLIGOSACCHARIDES
All values are given as R_{Glc}.

Fraction	*Mobilities in solvent*			
	A	*B*	*C*	*D*
F 1	0.03	0.34[a]	0.05	0.02
F 2	0.05	0.49	0.13	0.09
F 3	0.04	0.35[a]	0.08	0.01
F 4	0.05	0.49	0.08	0.01
F 5	0.07	0.63	0.18	0.03
F 6	0.13	0.55	0.18	0.26
F 7	0.18	0.68	0.21	0.29
F 8	0.05	0.13	0.08	0.19
F 9	0.34	0.73	0.17	0.28
F 10	1.47	1.35	1.95	1.02
F 11	1.09	1.00	1.67	0.63
Gal	0.89	0.93	0.93	0.91

[a] These oligosaccharides streaked and accurate values could not be obtained.

The results for the four solvent systems tested are shown in Table II. It is clear that neither of the two acid solvents C and D gave satisfactory separation of the oligosaccharides. Solvent C did however give good differentiation of the two carboxyethylidene derivatives of galactose from each other and from the free sugar, despite its poor resolution of the pyruvylated trisaccharide (F9) from the corresponding non-pyruvylated fragment. The neutral solvent A gave good separation of these two trisaccharides. For oligosaccharides which were acetylated or formylated, the solvent system of FISCHER AND DÖRFEL[8] gave good separation. This was not unexpected as it was originally used for the separation of amino sugars and the corresponding N-acetylated compounds. It was also observed that in this solvent system the acylated oligosaccharides gave discrete spots whereas glucuronic acid and aldobiouronic acids streaked. It thus appears that this solvent is the best of those tested for the separation of naturally acylated oligosaccharides of the type indicated in Table I.

The gift of *Salmonella typhimurium* polysaccharide from Dr. T. HOLME is gratefully acknowledged.

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1 I. W. SUTHERLAND, K. JANN AND B. JANN, *Eur. J. Biochem.*, 12 (1970) 285.
2 I. W. SUTHERLAND, *Biochem. J.*, 104 (1967) 278.
3 I. W. SUTHERLAND, *Nature*, 228 (1970) 280.
4 H. E. CONRAD, J. R. BAMBURG, J. D. EPLEY AND T. J. KINDT, *Biochemistry*, 5 (1966) 2808.
5 I. W. SUTHERLAND AND J. F. WILKINSON, *Biochem. J.*, 110 (1968) 749.
6 I. W. SUTHERLAND, *Biochem. J.*, 115 (1969) 935.
7 P. J. GAREGG, B. LINDBERG, T. ONN AND T. HOLME, *Acta Chem. Scand.*, (1971) in press.
8 F. G. FISCHER AND H. DÖRFEL, *Hoppe-Seyler's Z. Physiol. Chem.*, 302 (1955) 186.

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CHROM. 5355

Letter to the Editor

Dear Sir,

We wish to comment on the paper *Artifacts in Amino Acid Analysis. Ninhydrin-positive Products of Carbohydrate Hydrolysis*, by I. E. P. TAYLOR (*J. Chromatogr.*, 50 (1970) 331.

As early as 1953, SCHRAM *et al.*[1] described a red peak which eluted early in their amino acid analyses. They noted that foods containing starch, cellulose, glucose or lactose gave this anomalous peak with ninhydrin. It was shown that this peak (which they intimated might be levulinic acid) could be produced from carbohydrates in the absence of amino acids under conditions of acid hydrolysis. Subsequently, ZACHARIUS AND TALLEY[2] isolated a compound from acid hydrolysates of the non-protein nitrogen fraction of potato tubers and from bulk protein of kidney bean seeds that gave a similar red peak, which was unequivocally identified as levulinic acid. SCHILLING *et al.*[3] also identified the levulinic acid peak in amino acid chromatograms of crude protein hydrolysates.

Studying other anomalous peaks on amino acid analyzer chromatograms, ZACHARIUS AND PORTER[4] isolated from an unhydrolyzed extract of tart cherries material responsible for a high 440-nm peak with ninhydrin which they identified as fructose and glucose. The "positive" behavior of a large number of carbohydrates and related non-nitrogenous compounds with ninhydrin has been described[4–6].

More recently, products of the degradation of carbohydrates have been implicated to explain some anomalous peaks on the amino acid chromatograms of wood proteins[7]. A warning was extended on the potential hazard of interpreting peaks resulting from ninhydrin and high levels of carbohydrates and their hydrolytic products as products of imino acids[2,4,5].

In his paper, TAYLOR has indicated that some of his artifact peaks, which arose from the strong acid hydrolysis of pea seed coats, could also be demonstrated with sucrose under similar hydrolytic conditions. Such artifacts had also been demonstrated with nitrogen-free glucose and starch[2,8] using acid conditions similar[8] to those of TAYLOR. Although based on only the limited data provided, we conclude from our earlier work that TAYLOR's artifact peak No. 2 is almost certainly levulinic acid. The almost equal 440-nm and 570-nm absorbance of the ninhydrin reaction product is a characteristic of levulinic acid and some of its synthetic derivatives. While TAYLOR's artifact peak No. 1 might possibly be attributed to fructose, it and the remaining artifacts are more likely related to the isomeric angelicalactones, particularly products of the more unstable β,γ-angelicalactone[2]. The angelicalactones can be produced from levulinic acid.

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1 E. SCHRAM, J. P. DUSTIN, S. MOORE AND E. J. BIGWOOD, *Anal. Chim. Acta*, 9 (1953) 149.
2 R. M. ZACHARIUS AND E. A. TALLEY, *J. Chromatogr.*, 7 (1962) 51.

3 E. D. SCHILLING, P. I. BURCHILL, J. R. COFFMAN AND R. A. CLAYTON, *Biochem. Biophys. Res. Commun.*, 5 (1961) 217.
4 R. M. ZACHARIUS AND W. L. PORTER, *J. Chromatogr.*, 30 (1967) 190.
5 E. D. SCHILLING, P. I. BURCHILL AND R. A. CLAYTON, *Anal. Biochem.*, 5 (1963) 1.
6 C. L. HU, V. V. RENDIG AND E. A. MCCOMB, *J. Chromatogr.*, 19 (1965) 622.
7 G. SCURFIELD AND P. W. NICHOLLS, *J. Exp. Bot.*, 21 (1970) 857.
8 J. P. DUSTIN, C. CZAJKOWSKA, S. MOORE AND E. J. BIGWOOD, *Anal. Chim. Acta*, 9 (1953) 256.

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Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

HENSEL, I. B.: Chromatography in criminalistics. *Chem. Quart.*, 8, Nos. 3–4 (1967) 7–24; *C.A.*, 73 (1970) 33500y.

KAUFMANN, H. P.: Fortschritte auf dem Gebiet der Lipoidanalyse. II. Papier-Chromatographie. Teil 1. *Fette-Seifen-Anstrichmittel*, 72 (1970) 592–604 — a review with 163 references.

KAUFMANN, H. P.: Fortschritte auf dem Gebiet der Lipoidanalyse. III. Papier-Chromatographie. Teil 2. *Fette-Seifen-Anstrichmittel*, 72 (1970) 738–747 — a review with 181 references.

2. FUNDAMENTALS, THEORY AND GENERAL

DE LIGNY, C. L. AND VAN DE MEENT, W.: Peak broadening in paper chromatography and related techniques. VII. The contribution of the macroscopic mobile phase velocity profile to peak broadening in paper and thin-layer chromatography. *J. Chromatogr.*, 53 (1970) 469–475 — PC and TLC.

LITEANU, C. AND GOCAN, S.: Chromatographie sur papier avec gradient de température. *Bull. Soc. Chim. Fr.*, (1970) 4527–4539.

SOCZEWINSKI, E., CISZEWSKA, M. AND KUCZYNSKI, J.: Paper partition chromatography of very weak bases in systems of the type: weakly polar solvent–hydrochloric acid. II. *Bull. Acad. Pol. Sci., Ser. Sci. Chim.*, 18 (1970) 149–154; *C.A.*, 73 (1970) 29300r.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

WACHTER, H., GÜTTER, W., HAUSEN, A. AND SALLABERGER, G.: Ein einfaches Transferbesteck für Chromatographie und Elektrophorese. *Mikrochim. Acta*, (1970) 1022–1028 — PC and TLC.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

SEMENOV, A. D., BEISOVA, M. P. AND OBRAZTSOVA, M. M.: (Chromatographic luminescent method for determining petroleum products in natural waters). *Gidrokhim. Mater.*, 52 (1969) 127–132; *C.A.*, 73 (1970) 17579a.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

GELASHVILI, N. N., DZHEMUKHADZE, K. M. AND BUZUN, G. A.: (Determining grape catechols). *Vinodel. Vinograd. SSSR*, 30, No. 1 (1970) 21–22; *C.A.*, 73 (1970) 11235p.

NIKOLOV, N. AND IVANOV, V.; (Flavonoid composition of *Crataegus monogyna*. 1. Isolation of a flavonoid mixture from the leaves). *Farmatsiya (Sofia)*, 19, No. 6 (1969) 32–37; *C.A.*, 73 (1970) 32286w.

USMANOV, B. Z., KASYMOV, A. U. AND ABUBAKIROV, N. K.: (Spectrophotometric determination of furocoumarins from *Psoralea drupacea* and *Ficus carica*). *Khim. Prir. Soedin.*, 5 (1969) 473–476; *C.A.*, 73 (1970) 32208x.

9. OXO COMPOUNDS

WIDÉN, C. J., EUW, J. VON AND REICHSTEIN, T.: Trispara-aspidin, ein neues Phloroglucid aus dem Farn *Dryopteris remota* (A. Br.) Hayek. *Helv. Chim. Acta*, 53 (1970) 2176–2188 — PC and TLC.

See also TLC section.

10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

AMINOFF, D. AND FURUKAWA, K.: Enzymes that destroy blood groups specificity. I. Purification and properties of α-L-fucosidase from *Clostridium perfrigens*. *J. Biol. Chem.*, 245 (1970) 1659–1669.

NEY, K. H. AND WIROTAMA, I. P. G.: Untersuchung der Hydrolyse von Lactose. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 93–95 — PC and TLC.

11. ORGANIC ACIDS AND LIPIDS

11b. Lipids and their constituents

See TLC section.

13. STEROIDS

GOEBELSMANN, U. AND GORWILL, R. H.: The use of silica gel impregnated glass fiber sheets in plasma estriol assays. *Steroids*, 16 (1970) 635–647.

HAGEN, A. A. AND CONSROE, P. F.: Metabolism of [4-^{14}C]estradiol-17β by the newborn puppy. *Steroids*, 16 (1970) 723–731 — PC and TLC.

14. STEROID GLYCOSIDES AND SAPONINS

ELBANOWSKA, A.: (Quantitative determination of total cardenolides and convallatoxin in the blooms, leaves and galenical preparations of *Convallaria majalis*). *Herba Pol.*, 15 (1969) 211–215; *C.A.*, 73 (1970) 38591p.

MEISTER, L., KAUFMANN, H., STÖCKLIN, W. AND REICHSTEIN, T.: Die Glykoside der Wurzeln von Xysmalobium dilatatum Weimarck. 1. Isolierungen. *Helv. Chim. Acta*, 53 (1970) 1659–1677 — PC and TLC.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BOLSHAKOVA, T. D.: (Determination of methoxy derivatives of catecholamines in urine by means of paper chromatography). *Tr. Nov. App. Metod.*, No. 8 (1969) 109–117; *C.A.*, 73 (1970) 32180g.

NAKAI, T., UCHIJIMA, S. AND KOYAMA, M.: Paper chromatography of 3-methylhydantoic acid and 1-methylhydantoin, possible intermediates of microbial degradation of creatine and creatinine. *J. Chromatogr.*, 53 (1970) 406–408.

18. AMINO ACIDS

KLEIN, A., CHUDZIK, J. AND SARNECKA-KELLER, M.: The application of thin-layer electrophoresis and chromatoelectrophoresis on Sephadex G-25 to the analysis of amino acid and peptide composition of biological fluids. *J. Chromatogr.*, 53 (1970) 329–336.

LUCHI, P. AND DE MARCO, C.: Thiosulfate production during transamination of S-sulfohomocysteine. *Ital. J. Biochem.*, 18 (1969) 451–456; *C.A.*, 73 (1970) 31606p.

SIMMONS, W. K.: Minimal amount of serum necessary for the determination of the plasma amino acid ratio. *Bull. WHO*, 42 (1970) 484–486; *C.A.*, 73 (1970) 32170d.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

JOLLÈS, J., ALAIS, CH. AND JOLLÈS, P.: Chemical structure studies of cow $\varkappa$-casein: Study of the soluble tryptic peptides. *Helv. Chim. Acta*, 53 (1970) 1918–1926.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

HOLÝ, A.: Oligonucleotide compounds. XXXVIII. Synthesis of diribonucleoside phosphates and triribonucleoside diphosphates derived from adenosine 3′-phosphate and guanosine 3′-phosphate: A general approach to the synthesis of oligonucleotides. *Coll. Czech. Chem. Commun.*, 35 (1970) 3686–3711 — R_F values for 189 compounds.

WATANABE, M. S., MCCROSKEY, R. P. AND ABBOTT, M. T.: The enzymatic conversion of 5-formyluracil to uracil-5-carboxylic acid. *J. Biol. Chem.*, 245 (1970) 2023–2026.

22. ALKALOIDS

LUTOMSKI, J. AND WISNIEWSKI, J.: (Estimation of glaucine hydrochloride stability in injection solutions). *Herba Pol.*, 15 (1969) 216–221; *C.A.*, 73 (1970) 18498k.

PEKIC, B., PETROVIC, K. AND GORUNOVIC, M.: (Determination of hyoscyamine and scopolamine by paper and thin-layer chromatography). *Arch. Farm.*, 19 (1970) 235–244; *C.A.*, 73 (1970) 18499m — PC and TLC.

See also TLC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BELCHER, R. V., SMITH, S. G., MAHLER, R. AND CAMPBELL, J.: The separation of free dicarboxylic acid porphyrins using thin-layer and paper chromatography. *J. Chromatogr.*, 53 (1970) 279–284 — PC and TLC on talc.

24. ORGANIC SULPHUR COMPOUNDS

GMELIN, R. AND SCHLÜTER, M.: Isolierung von 4-Methylthiobutylglucosinolat (Glucoerucin) aus Samen von *Eruca sativa* Mill. *Arch. Pharm.*, 303 (1970) 330–334 — PC and TLC.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

KELLER-SCHIERLEIN, W. AND DIEKMANN, H.: Stoffwechselprodukte von Mikroorganismen. 85. Mitt. Zur Konstitution des Coprogens. *Helv. Chim. Acta*, 53 (1970) 2035–2044 — PC and TLC.

27. VITAMINS AND VARIOUS GROWTH FACTORS

See TLC section.

28. ANTIBIOTICS

CARR, J. P., STRETTON, R. J. AND WATSON-WALKER, J.: Chromatographic separation of neomycin, polymyxin B and bacitracin. *Loughborough Univ. Technol., Dep. Chem., Sum. Final Year Stud. Proj. Theses*, 10 (1969) 17–18; *C.A.*, 73 (1970) 28954v — PC and TLC.

DE FRANCA, F. P., ROSEMBERG, J. A. AND DE JESUS, A. M.: (Quantitative determination of tyrothricin by ascending paper chromatography). *Rev. Brasil. Farm.*, 50 (1969) 343–346; *C.A.*, 73 (1970) 28952t.

29. INSECTICIDES AND OTHER PESTICIDES

BILIBINA, V. S.: (Use of circular paper chromatography for analysing the fungicide 3,3-ethylenebis(4,6-dimethyltetrahydro-1,3,5-thiadiazine-2-thione) and its decomposition products). *Gig. Sanit.*, 35, No. 2 (1970) 61–63; *C.A.*, 73 (1970) 24176v.

30. SYNTHETIC AND NATURAL DYES

KARLE KESZTHELY, A.: (Chromatographic investigation on basic dyes). *Kolor. Ert.*, 12, No. 1–2 (1970) 32–40; *C.A.*, 73 (1970) 16246c.

SCHLEGELMILCH, F. AND FUCHS, M.: (Chromatographic dye analysis. 2. Paper chromatographic analysis of acid azo dyes. 1. Paper chromatographic analysis of metal complex dyes). *Z. Ges. Textilind.*, 72 (1970) 388–393; *C.A.*, 73 (1970) 41617g.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

IONESCU-STOIAN, P., SAVOPOL, E., MIHAILESCU, F. AND IONICA, V.: (Interaction of excipients with drugs. V. Chromatographic behaviour of several drugs in the presence of non-ionic macromolecular agents). *Farmacia (Bucharest)*, 18 (1970) 143–147; *C.A.*, 73 (1970) 28882v.

MAES, R., GIJBELS, M. AND LARUELLE, L.: A rapid chromatographic procedure for the detection of some diuretics in pharmaceuticals and biological fluids. *J. Chromatogr.*, 53 (1970) 408–412.

33. INORGANIC SUBSTANCES

ABE, S. AND WEISZ, H.: The rapid separation and determination of uranium (VI) by use of DEAE anion-exchange paper and the ring-oven technique. *Mikrochim. Acta*, (1970) 550–552.

PRZESZLAKOWSKI, S.: (Chromatography of some ions on paper impregnated with hydrofluoric acid by using benzene solutions of liquid anion exchanger as mobile phase). *Rocz. Chem.*, 44 (1970) 269–278; *C.A.*, 73 (1970) 38910s.

THIELEMANN, H.: Zur dünnschicht- und papierchromatographischen Trennung der Cyanid-, Thiocyanat- und Carbonationen. *Mikrochim. Acta*, (1970) 645 — PC and TLC.

VDOVENKO, M. E. AND KAPLAN, M. L.: (Separating platinum group metals from base metals). *USSR Pat.*, 263, 984 (Cl. G 01n), 10 Feb. 1970, Appl. 11 Apr. 1968; *C.A.*, 73 (1970) 27829w.

See also TLC section.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

CHAIN, E. B., LOWE, A. E. AND MANSFORD, K. R. L.: A computerised scanner for bidimensional radiochromatograms. *J. Chromatogr.*, 53 (1970) 293–314.

FALECKI, J.: Purification of synthetic ^{14}C-labeled compounds by the use of preparative paper chromatography. *J. Label. Compounds*, 6 (1970) 15–20; *C.A.*, 73 (1970) 24433b.

FISCHER, E.: Untersuchungen mit ^{14}C-markiertem Pyrokohlensäurediethylester. II. Mitt. Reaktionen mit Vitaminen und anderen Lebensmittelbestandteilen. *Z. Lebensm. Untersuch. Forsch.*, 144 (1970) 262–268.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

BERÁNEK, E.: (Isolation and separation of oxidation products of motor oils). *Ropa Uhlie*, 12 (1970) 177–185; *C.A.*, 73 (1970) 27232w.

LAHAM, S. AND FARANT, J. P.: Identification of bladder carcinogens by paper chromatography. *Ind. Med. Surg.*, 39 (1970) 142–147; *C.A.*, 73 (1970) 21999e.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

KAUFMANN, H. P.: Fortschritte auf dem Gebiet der Lipoid-Analyse. IV. Dünnschicht-Chromatographie. Teil 1. *Fette-Seifen-Anstrichmittel*, 72 (1970) 811–825 — a review with 204 references.

KAUFMANN, H. P.: Fortschritte auf dem Gebiet der Lipoid-Analyse. V. Dünnschicht-Chromatographie. Teil 2. *Fette-Seifen-Anstrichmittel*, 72 (1970) 902–915 — a review with 188 references.

NIEDERWIESER, A. AND PATAKI, G. (Editors): *Progress in Thin-layer Chromatography and Related Methods*, Vol. 1, Ann Arbor-Humphrey, Ann Arbor, Mich., 1970, 240 pp.; *C.A.*, 73 (1970) 38979w.

2. FUNDAMENTALS, THEORY AND GENERAL

HAGYMASSY, JR., J. AND BRUNAUER, S.: Pore structure analysis by water vapor adsorption. II. Analysis of five silica gels. *J. Colloid Interface Sci.*, 33 (1970) 317–327; *C.A.*, 73 (1970) 38921w.

NETTING, A. G.: Column separations designed precisely with the aid of thin-layer chromatography. *J. Chromatogr.*, 53 (1970) 507–516.

VOYTUS, W. A.: Chromatographic systems analysis: moment analysis of the equilibrium adsorption model. *NASA Contract. Rep.*, NASA-CR-106230 (1969) 26 pp.; *C.A.*, 73 (1970) 18816n.

See also PC section.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

GEISS, F.: Letter to the editor concerning paper by DE ZEEUW, R. A. *et al.*: Improved separation in thin-layer chromatography using a single-component solvent in an unsaturated chamber [*J. Chromatogr.*, 47 (1970) 382]. *J. Chromatogr.*, 53 (1970) 620–622.

HASHMI, M. H., CHAUDRY, M. A., CHUGHTAI, N. A. AND RIAZ-UR-REHMAN: The effect of various factors on micro-analysis by circular thin-layer chromatography. *Mikrochim. Acta*, (1970) 200–206.

HUMPHREY, A. M.: The examination of volatile oils by combined gas–liquid chromatography/thin-layer chromatography. *J. Chromatogr.*, 53 (1970) 375–378.

JANÁK, J., JAGARIĆ, Z. AND DRESSLER, M.: Use of olefin–Ag^+ complexes for chromatographic separations of higher olefins in liquid–solid systems. *J. Chromatogr.*, 53 (1970) 525–530.

MUNSON, A. K., MUELLER, J. R. AND YANNONE, M. E.: Inexpensive sample applicator for thin-layer chromatography. *Microchem. J.*, 15 (1970) 95–96; *C.A.*, 73 (1970) 11208g.

NAU, H.: Empfindlicher Nachweis von Östrogenen mit einem automatischen Leitfähigkeitsdetektor für die Dünnschicht-Chromatographie. *J. Chromatogr.*, 53 (1970) 391–394 — 20 ng is detectable on thin layers, 0.2 ng on thin films.

OVERGAARD NIELSEN, M.: (Thin-layer chromatography on microscope slides). *Dan. Tidsskr. Farm.*, 44 (1970) 7–15; *C.A.*, 73 (1970) 28942q.

RAMOS, V. B.: Use of glass rods as support of the adsorbent in thin-layer chromatography. *Chem. Quart.*, 8, No. 3–4 (1967) 30–60; *C.A.*, 73 (1970) 18814k.

RÖDER, E.: Fliessmittelgemische aus isobaren Komponenten zur Dünnschicht-Chromatographie. *Mitt. Deut. Pharm. Ges. (Beil. Arch. Pharm.)*, 40 (1970) 176–180.

STAHL, E. AND SCHILD, W.: Schnell-Transfer Dünnschichtchromatographie–Infrarotspektroskopie. *J. Chromatogr.*, 53 (1970) 387–389.

See also PC section.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

CHAHL, J. S. AND KRATZING, C. C.: Simple apparatus for preparative and quantitative thin-layer chromatography. *Lab. Pract.*, 19 (1970) 289–290; *C.A.*, 73 (1970) 11230h.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

DIETSCHE, W.: Dünnschicht-Chromatographie von Kohlenwasserstoff-Wachsen an harnstoffbeschichteten Platten. *Fette-Seifen-Anstrichmittel*, 72 (1970) 778–783.

RAAEN, H. P.: Chromatography and 77°K luminescence of some hydrocarbons on thin layers of microcrystalline nylon–polytetrafluoroethylene (Aviamide-6-Fluoroglide 200). *J. Chromatogr.*, 53 (1970) 600–604.

7. PHENOLS

GHIGLIONE, C., PUGNET, T. AND GIRAUD, M.: (Thin-layer chromatography of hydroxyazo derivatives. Application to the determination of phenols). *Bull. Soc. Pharm. Marseille*, 18 (1969) 91–98; *C.A.*, 73 (1970) 28975c.

KIMURA, K., SHIMADA, H., NOMURA, S., HISADA, Y. AND TANAKA, T.: Quality control of crude drugs in Japanese Pharmacopeia. I. Determination of arbutin. *Yakugaku Zasshi*, 90 (1970) 394–397; *C.A.*, 73 (1970) 18504j.

MAY, C. E. AND BROWN, J. M. A.: The separation of plant glycosides and aglycones using thin-layer chromatography and electrophoresis. *J. Chromatogr.*, 53 (1970) 399–402.

THIELEMANN, H.: Zur dünnschichtchromatographischen Trennung der Kondensationsprodukte des *o*-,*m*-,*p*-Kresols, Phenols (*p*-Benzochinons) und des 1- und 2-Naphthols mit 1-Phenyl-2,3-dimethyl-4-aminopyrazolon-(5). *Mikrochim. Acta*, (1970) 994–995.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

CUCULLU, A. F., LEE, L. S., PONS, JR., W. A. AND GOLDBLATT, L. A.: Determination of aflatoxins in peanut and cottonseed soapsocks. *J. Amer. Oil Chem. Soc.*, 47 (1970) 226–228.

HAAG, T. P.: Zum Nachweis von Rauschgiften, insbesonders Haschisch. *Deut. Apotheker-Ztg.*, 110 (1970) 1874–1877.

KAUFMANN, H. P. AND EL BAYA, A. W.: Pro- und Antioxydantien auf dem Fettgebiet. XXVI. Anthochlore von *Dahlia variabilis* und *Cosmos sulphureus*. *Fette-Seifen-Anstrichmittel*, 72 (1970) 372–376.

KIERMEIER, F.: Zum Nachweis von Aflatoxinen in Käse. *Z. Lebensm. Untersuch. Forsch.*, 144 (1970) 293–297.

MAING, I. Y., LILLARD, H. S. AND AYRES, J. C.: The rapid separation of aflatoxins on silica gel coated glass cylinders. *J. Amer. Oil Chem. Soc.*, 47 (1970) 212–214.

MURKO, D. AND JANKOVIC, M.: (Thin-layer chromatography of commercial tannins). *Kem. Ind.*, 18 (1969) 730–731; *C.A.*, 73 (1970) 41627k.

STICHER, O.: Galiridosid, ein Iridoidglucosid aus *Galeopsis tetrahit* L. (Labiatae). *Helv. Chim. Acta*, 53 (1970) 2010–2020.

STUBBLEFIELD, R. D., SHANNON, G. M. AND SHOTEWELL, O. L.: Aflatoxins M_1 and M_2: preparation and purification. *J. Amer. Oil Chem. Soc.*, 47 (1970) 389–390.

VÖLKSEN, W.: Hanf als Arzneimittel und Rauschdroge. *Deut. Apotheker-Ztg.*, 110 (1970) 1869–1873.

YEN, K. Y., CHANG, B. F., YANG, L. L. AND PAN, M. H.: Chemical constituents of umbelliferous plants of Taiwan. X. Determination of coumarin compounds by TLC. *T'ai-wan Yao Hsueh Tsa Chih*, 19, No. 12 (1967) 28–37; *C.A.*, 73 (1970) 11381h.

9. OXO COMPOUNDS

CHRISTOL, H., PLÉNAT, F. AND SANCON, J.: Condensation d'aldoénamines cyclaniques avec la méthylvinylcétone. *Bull. Soc. Chim. Fr.*, (1970) 4468–4471.

FRANZKE, C., STROBACH, J. AND SCHILLING, B.: Zur Bildung von Ketonen und Aldehyden während der Oxydation gesättigter Triglyceride bei hohen Temperaturen. *Fette-Seifen-Anstrichmittel*, 72 (1970) 629–635.

ŠVEC, P., SEIFERT, R. AND ZBIROVSKÝ, M.: Chromatographic studies of some halogenated quinones. I. Thin-layer chromatography of some chlorinated derivatives of *p*-benzoquinone. *J. Chromatogr.*, 53 (1970) 587–589.

SZENDEY, G. L. AND NOOK, L. G.: Die Cyanessigesterreaktion substituierter Naphthochinone. 1. Mitt. Die spektrophotometrische Bestimmung von Menadion. *Deut. Apotheker-Ztg.*, 110 (1970) 2038–2039 — TLC and PC.

See also PC section.

10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

ABELLI, G. AND FALAGARIO, M.: (Carbohydrate composition of normal human seminal fluid. Thin-layer chromatography). *Attual. Ostet. Ginecol.*, 14 (1968) 391–395; *C.A.*, 73 (1970) 33030v.

BOTT, D. E., HOPLEY, P. J. AND LEACH, R. H.: Studies with Caloreen, a glucose polymer mixture. *Pharm. J.*, 204 (1970) 583–584; *C.A.*, 73 (1970) 38489m.

CRÖSSMANN, F., KUPFER, H., SOUCI, S. W. AND MERGENTHALER, E.: Zur physikalischen und chemischen Charakterizierung der als Lebensmittel-Zusatzstoffe verwendeten Celluloseäther. IV. Mitt. Verfahren zum Nachweis und zur Bestimmung von Celluloseäthern in Lebensmitteln. *Z. Lebensm. Untersuch. Forsch.*, 142 (1970) 410–418.

NAGASAWA, K., OGAMO, A., HARADA, H. AND KUMAGAI, K.: Detection of sugar derivatives by perchloric acid on cellulose thin layers. *Anal. Chem.*, 42 (1970) 1436–1438.

See also PC section.

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

TANIGUCHI, N.: Thin-layer gel filtration of urinary acid mucopolysaccharides in Hunter's syndrome and in normal individuals. *Clin. Chim. Acta*, 30 (1970) 801–808.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

CANIC, V. D. AND PERISIC-JANJIC, N.: (Separation of organic acids by thin-layer chromatography on starch). *Tehnika (Belgrade)*, 25 (1970) 330–332; *C.A.*, 73 (1970) 31374m.

DAVÍDKOVÁ, E., DAVÍDEK, J. AND KUČEROVÁ, Z.: Beurteilung der Methoden zur Bestimmung freier Fettsäuren in Gewebelipiden. *Z. Lebensm. Untersuch.-Forsch.*, 144 (1970) 89–91.

FIDELUS, J. AND ZIETEK, M.: Eine indirekte polarographische Bestimmung von 2,4-Dichlorphenoxyessigsäure in biologischem Material. *Mikrochim. Acta*, (1970) 1010–1016.

FREEDMAN, B.: Gas–liquid and thin-layer chromatographic analysis of 12-aminostearic acid. *J. Amer. Oil Chem. Soc.*, 47 (1970) 305–310.

PARIS, R. R. AND NISHIO, M.: (Separation of the chlorogenic acid of green coffee, *Coffea robusta*). *C. R. Acad. Sci., (Paris), Ser. D*, 270 (1970) 1465–1467; *C.A.*, 73 (1970) 32171e.

WOIDICH, H., GNAUER, H., GALINOVSKY, E. AND RIEDL, O.: Dünnschichtchromatographischer Nachweis von Elaidinsäure in Speisefetten. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 175–179.

11b. Lipids and their constituents

ABELLI, G., DOMINI, E., FALAGARIO, M. AND CARLASSARE, F.: (Thin-layer chromatography of phospholipids in Warton's jelly from the human umbilical cord at the term of psychological pregnancy). *Attual. Ostet. Ginecol.*, 14 (1968) 37–41; *C.A.*, 73 (1970) 33028a.

BURKHARDT, H. J.: The phosphatides of safflower seeds involved in color formation occurring in extracted and heated crude oils. *J. Amer. Oil Chem. Soc.*, 47 (1970) 69–72 — TLC and PC.

DIMITRIJEVIC, M., STOJNOVIC, N. AND DIMITRIJEVIC, N.: (Direct densitometric determination of serum lipids separated by thin-layer chromatography). *Acta Vet.*, 19 (1969) 205–207; *C.A.*, 73 (1970) 21992x.

GRYNBERG, H. AND CEGLOWSKA, K.: (Study of the glyceride composition of vegetable oils by thin-layer chromatography on silver nitrate-impregnated silica. II. Analysis of *Crambe abyssinica* oil). *Rev. Fr. Corps Gras*, 17 (1970) 173–176; *C.A.*, 73 (1970) 26889d.

KLEINING, H. AND LEMPERT, U.: Phospholipid analysis on a micro scale. *J. Chromatogr.*, 53 (1970) 595–597.

MCKILLICAN, M. E. AND LAROSE, J. A. G.: Residual lipids of hexane-extracted rapeseed meal. *J. Amer. Oil Chem. Soc.*, 47 (1970) 256–258 — TLC and PC.

MOUSA, A. H., ATTA, A., AHMAD, A. K. S., WAHAB, M. F. A., RADWAN, S. S. AND MADWAR, M. A.: The lipids of schistosomes during their various stages of growth. *Fette-Seifen-Anstrichmittel*, 72 (1970) 13–16.

NAGAI, J. AND HIGUCHI, K.: Quantitative analysis of serum phospholipids by thin-layer chromatography. *Rinsho Byori*, 18 (1970) 9–12; *C.A.*, 73 (1970) 32179p.

SHEPHERD, G. F., BURNS, D. T. AND STRETTON, R. J.: Synergistic complexation effects in the thin-layer chromatography of certain triglycerides on silica gel impregnated with silver nitrate. *Loughborough Univ. Technol. Dep. Chem., Sum. Final Year Stud. Proj. Theses*, 10 (1969) 61–63; *C.A.*, 73 (1970) 38948k.

SNYDER, F., BLANK, M. L., MALONE, B. AND WYKLE, R. L.: Identification of O-alkyldihydroxyacetone phosphate, O-alkyldihydroxyacetone and diacylglyceryl ethers after enzymic synthesis. *J. Biol. Chem.*, 245 (1970) 1800–1805.

SNYDER, F., MALONE, B. AND BLANK, M. L.: Enzymic synthesis of O-alkyl bonds in glycerolipids. *J. Biol. Chem.*, 245 (1970) 1790–1799.

SOULA, G., SOUILLARD, C., MAURY, Y. AND DOUSTE-BLAZY, L.: Étude chromatographique des stérides et des esters méthyliques des noyaux d'hématies nucléées. *J. Chromatogr.*, 53 (1970) 217–224.

STEFANOV, K. AND POPOV, As.: (Phosphorus determination in polar lipids). *Dokl. Bulg. Akad. Nauk*, 23 (1970) 165–167; *C.A.*, 73 (1970) 31382n.

WEBER, E. J.: Lipids of maturing grain of corn (*Zea mays* L). II. Changes in polar lipids. *J. Amer. Oil. Chem. Soc.*, 47 (1970) 340–343.

13. STEROIDS

ANASTASINA, G. V.: (Quantitative determination of aldosterone in daily urine by thin-layer chromatography). *Tr. Nov. App. Metod.*, No. 8 (1969) 37–43; *C.A.*, 73 (1970) 21750s.

GALLETTI, F.: Estimation of urinary testosterone and epitestosterone by thin-layer chromatography and fluorimetry. *Res. Steroids*, 3 (1968) 155–158; *C.A.*, 73 (1970) 10994y.

GANJAM, V., DESJARDINS, C. AND EWING, L. L.: A quantitative procedure for the determination of cortisol and corticosterone in blood plasma. *Steroids*, 16 (1970) 227–250.

GOSWAMI, S. K. AND FREY, C. F.: Manganous chloride spray reagent for cholesterol and bile acids on thin-layer chromatograms. *J. Chromatogr.*, 53 (1970) 389–390.

KARTNIG, TH. AND MIKULA, G.: Zur Identifizierung und Bestimmung von Sterolen. *J. Chromatogr.*, 53 (1970) 537–543.

KAUFMANN, H. P., VENNEKEL, E. AND HAMZA, Y.: Über die Veränderung der Sterine in Fetten und Ölen bei der industriellen Bearbeitung derselben. I. *Fette-Seifen-Anstrichmittel*, 72 (1970) 242–246.

KUSHINSKI, S., COYOTUPA, J., HONDA, K., HIROI, M., KINOSHITA, K., FOOTE, M., CHAN, C., HO, R. Y., PAUL, W. AND DIGNAM, W. J.: Gas chromatographic determination of estrone, estradiol and estriol in non-pregnancy plasma. *Mikrochim. Acta*, (1970) 491–503.

MASSA, V.: (Chromatography and photodensitometry of some antiinflammatory corticoids. Application to the control of ointments). *Labo-Pharma-Probl. Tech.*, 18 (1970) 80–81; *C.A.*, 73 (1970) 38585q.

MUJTABA NAQVI, S. H. AND NICHOLAS, H. J.: Conversion of 3-keto-5β-cholanoic acid to lithocholic acid by guinea-pig brain tissue, *in vitro*. *Steroids*, 16 (1970) 297–316.

SCHREIBER, J., ERB, W. AND BOEHLE, E.: (Analysis of foecal bile acids). *Verh. Deut. Ges. Inn. Med.*, 75 (1969) 920–923; *C.A.*, 73 (1970) 11234n.

SOMMERVILLE, I. F., FERNANDEZ, G. E., MAROCCHI, A. AND SHARPLES, M. J.: Aspects of the determination of testosterone and epitestosterone by thin-layer and gas–liquid chromatography. *Res. Steroids*, 3 (1968) 185–192; *C.A.*, 73 (1970) 10996a.

SZEKACS, I. AND KLEMBALA, M.: (Thin-layer chromatographic separation of oral contraceptives and some sex steroids). *Z. Klin. Chem. Klin. Biochem.*, 8 (1970) 131–133; *C.A.*, 73 (1970) 31792e.

See also PC section.

14. STEROID GLYCOSIDES AND SAPONINS

KARTNIG, TH. AND DANHOFER, R.: Zur Identifizierung und quantitativen Analyse von Strophantusglykosiden. *J. Chromatogr.*, 52 (1970) 313–320.

VLASENKO, L. M.: (Isolation, detection and determination of oleandrin in chemical and toxicological studies). *Farmatsiya (Moscow)*, 19, No. 2 (1970) 53–58; *C.A.*, 73 (1970) 33503b.

WOLF, W. J. AND THOMAS, B. W.: Thin-layer and anion-exchange chromatography of soybean saponins. *J. Amer. Oil Chem. Soc.*, 47 (1970) 86–90.

See also PC section.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

AUF DEM KELLER, S., PACHALY, P. AND ZYMALKOWSKI, F.: Ursolsäure und Uvaol als Inhaltsstoffe von *Rhododendron ponticum*. *Arch. Pharm.*, 303 (1970) 243–248.

MUNOZ TERAN, O.: (Thin-layer chromatographic identification of essential oils in pomades, ointments and solutions). *Rev. Fac. Quim. Farm.*, (*Univ. Cent. Ecuador*), 5, No. 9 (1966) 67–71; *C.A.*, 73 (1970) 38430k.

POZZO-BALBI, T. AND NOBILE, L.: (Composition of the essential oil of *Citrus trifoliata* fruit peels. II). *Ann. Chim.* (*Rome*), 60 (1970) 171–187; *C.A.*, 73 (1970) 38427q.

YAMAMOTO, S. AND BLOCH, K.: Studies on squalene epoxidase of rat liver. *J. Biol. Chem.*, 245 (1970) 1670–1674.

16. NITRO AND NITROSO COMPOUNDS

ROSSEEL, M.-T., BOGAERT, M. G. AND MOERMAN, E. J.: Quantitative analysis of glyceryl nitrates on thin-layer chromatograms. Comparison of colorimetry and densitometry. *J. Chromatogr.*, 53 (1970) 263–268 — flying-spot densitometer.

VENTERS, K., APSITE, M. AND LOLYA, D. O.: (Thin-layer chromatography of furan nitration products). *Latv. PSR Zinat. Akad. Vestis*, No. 2 (1970) 10–17; *C.A.*, 73 (1970) 31375n.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

AHARONI, S. M. AND LITT, M. H.: Cobalt(II) as a developing agent in thin-layer chromatography of amine derivatives. *Anal. Chem.*, 42 (1970) 1467–1468.

CAVALLARO, A. AND ROSSI, G.: Thin-layer chromatographic analysis of arylamines and phenols used in oxidative hair dyes. I. Separation and identification. *Boll. Chim. Farm.*, 109 (1970) 25–32; *C.A.*, 73 (1970) 28765j.

RANGOONWALA, R. AND SEITZ, G.: "*cis*-Capsaicin". Ein Beitrag zur Synthese, Analytik und dem vermuteten Vorkommen in Capsicum-Früchten. *Deut. Apotheker-Ztg.*, 110 (1970) 1946–1949.

SKELLY, N. E.: Identification and determination of impurities in salicylanilide. *Anal. Chim. Acta*, 49 (1970) 267–274.

ULRICH, G.: Die analytische Beurteilung von Tribromsalicylanilid. *Fette-Seifen-Anstrichmittel*, 72 (1970) 199–201.

VEIBEL, S.: Characterization of amidines as picrates through thin-layer chromatography. *Anal. Chim. Acta*, 51 (1970) 309–315 — R_F values for 19 compounds.

18. AMINO ACIDS

ARNETH, W. AND HAMM, R.: Über die Hydroxyprolinbestimmung zur Ermittlung des Kollagenen Bindengewebes in Fleisch und Fleischerzeugnissen. I. Mitt. Unterschiede im Reaktionsablauf bei Oxydation mit Wasserstoffperoxid und Chloramin T. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 161–166.

CONSTANTINESCU, A. AND NEGOESCU, I.: Method for the separation of thyroid hormones and their metabolites by circular thin-layer chromatography. *Rev. Roum. Endocrinol.*, 7 (1970) 79–83; *C.A.*, 73 (1970) 31966f.

GRÜNER, K.: (Identification of PTH amino acids by thin-layer chromatography on Silufol). *Chem. Listy*, 64 (1970) 1160–1171.

MAUGENET, J. AND DALMEYDA, M. L.: (Thin-layer two-dimensional chromatographic separation of amino acids on film). *Ann. Technol. Agr.*, 18 (1969) 237–240; *C.A.*, 73 (1970) 41620c.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

SIEBER, P., RINIKER, B., BRUGGER, M., KAMBER, B. AND RITTEL, W.: Menschliches Calcitonin. VI. Die Synthese von Calcitonin M. *Helv. Chim.Acta*, 53 (1970) 2135–2150 — R_F values for 27 compounds.

19b. Elucidation of structure of proteins

KUNDU, N. AND ROY, S.: Separation of dansylated products of L-seryltyrosine and their identification. *J. Chromatogr.*, 53 (1970) 598–600.

20. PROTEINS (INCLUDING ENZYMES)

COPIUS-PEEREBOOM, J. W.: Studies on alkaline milk phosphatase. III. A theory for the mechanism of reactivation of alkaline milk phosphatase in a model system. *Fette-Seifen-Anstrichmittel*, 72 (1970) 299–308 — TLC on Sephadex G-200.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

BALTES, W.: Quantitative Bestimmung von Inosinsäure in Fleischextrakten und Fertigsuppen-Erzeugnissen mittels Remissions-Spektralphotometrie. *Z. Lebensm. Untersuch. Forsch.*, 144 (1970) 305–308.

22. ALKALOIDS

AGWADA, V., PATEL, M. B., HESSE, M. AND SCHMID, H.: Alkaloide. 139. Mitt. Die Alkaloide aus *Hedranthera barteri* (Hook. f.) Pichon. *Helv. Chim. Acta*, 53 (1970) 1567–1577.

BÖHME, H. AND BITSCH, R.: Dünnschichtchromatographische Bestimmung von Chinin in Chinarinden. *Arch. Pharm.*, 303 (1970) 418–427.

BÖHME, H. AND BITSCH, R.: Schichtchromatographische Trennung und Reinigung von China-Alkaloiden. *Arch. Pharm.*, 303 (1970) 456–461.

GRAF, E. AND RÖNSBERG, W.: Eine neue Methode zur Bestimmung der Gesamtalkaloide und des Cephaelins in Ipecacuanha. *Arch. Pharm.*, 303 (1970) 209–217.

JANICKI, S.: (Spray-dried alkaloid extracts. II. Chromatographic analysis of spray-dried extracts). *Herba Pol.*, 15 (1969) 239–246; *C.A.*, 73 (1970) 28993g.

KIRICHENKO, L. O. AND KAGAN, F. YU.: (Chromatospectrophotometric method for the quantitative estimation of theophylline, dimedrol and ephedrine hydrochloride in drugs). *Farm. Zh. (Kiev)*, 25, No. 1 (1970) 42–47; *C.A.*, 73 (1970) 28951s.

MIRZAZADE, N. A.: (Quantitative determination of reserpine in some preparations by thin sorbent layer chromatography). *Azerb. Med. Zh.*, 47, No. 2 (1970) 26–30; *C.A.*, 73 (1970) 38588t.

NACKEN, M., PACHALY, P. AND ZYMALKOWSKI, F.: Aminoacylierungen des Emetins. *Arch. Pharm.*, 303 (1970) 122–133.

PASICH, B., KOWALEWSKI, Z. AND SKIBICKI, J.: Spectrophotometric method of assay of solasodine in the leaves of *Solanum laciniatum* (*aviculare*). *Diss. Pharm. Pharmacol.*, 22 (1970) 41–47; *C.A.*, 73 (1970) 38583n — TLC and PC.

POLESUK, J. AND MA, T. S.: Microchemical investigation of medicinal plants. VIII. Separation and identification of tropane alkaloids by thin-layer chromatography. *Mikrochim. Acta*, (1970) 670–676 — R_F values for 10 alkaloids.

POLESUK, J. AND MA, T. S.: Organic synthesis on the microgram scale. VI. Nitration and dehydration of the tropane alkaloids. *Mikrochim. Acta*, (1970) 677–682.

WASHÜTTL, J., BANCHER, E. AND RIEDERER, P.: Eine neue dünnschichtchromatographische Methode zur Bestimmung des Coffeingehaltes in gerösteten Kaffeebohnen. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 253–256.

See also PC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

FREI, R. W., KUNZ, A., PATAKI, G., PRIMS, T. AND ZÜRCHER, H.: The determination of nicotinic acid and nicotinamide by thin-layer chromatography and *in situ* fluorimetry. *Anal. Chim. Acta*, 49 (1970) 527–534.

HUMBEL, R.: Thin-layer chromatography of urinary imidazoles. *Clin. Chem.*, 16 (1970) 58–59; *C.A.*, 73 (1970) 11219m.

WASHÜTTL, J.: Ein neue dünnschichtchromatographische Bestimmungsmethode für Nicotinsäure und Nicotinsäureamid auf Grund der Fleckengrösse. *Mikrochim. Acta*, (1970) 621–627.

See also PC section.

24. ORGANIC SULPHUR COMPOUNDS

HASHMI, M. H., CHUGHTAI, N. A., AHMAD, I. AND AFZAL, A. U.: Semiquantitative determination of thiourea, thiocyanate and sulfide by circular thin-layer chromatography. *Mikrochim. Acta*, (1970) 254–257.

PÜSCHEL, F. AND PRESCHER, D.: Dünnschichtchromatographie von Sulfonsäureestern. *J. Chromatogr.*, 53 (1970) 395–396.

See also PC section.

25. ORGANIC PHOSPHORUS COMPOUNDS

POTAPOV, A. M., KRASIL'NIKOVA, E. A., RAZUMOV, A. I. AND BAIGIL'DINA, S. YU.: (Chromatographic behaviour of some organothiophosphorus compounds of tri- and pentavalent phosphorus). *Zh. Obshch. Khim.*, 40 (1970) 564–566; *C.A.*, 73 (1970) 29303u.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

HIROKI, S., NAKANO, T. AND KOBAYASHI, H.: Analysis of zinc dialkyl dithiophosphates by thin-layer chromatography. *Sekiyu Gakkai Shi*, 13 (1970) 124–126; *C.A.*, 73 (1970) 27230u.

See also PC section.

27. VITAMINS AND VARIOUS GROWTH FACTORS

HOUSTON, M. R., MCVEIGH, I. AND PEARSON, W. N.: Bioautographic procedure for detection of thiamine metabolites by use of *Phycomyces blakesleeanus*. *Mycologia*, 61 (1969) 1168–1171; *C.A.*, 73 (1970) 32177m.

KOVÁTS, L. T., KRASZNER-BERNDORFER, E., PÉTERFALVI, M. AND GÁBOR, T.: Zum natürlichen Vorkommen der Pangaminsäure (Vitamin B_{15}). I. Mitt. Pangaminsäuregehalt von Getreide. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 411–418.

OHMAE, M. AND KATSUI, G.: Photolysis of vitamin K_1. V. Photolytic products in the absence of oxygen. *Bitamin*, 41 (1970) 178–184; *C.A.*, 73 (1970) 28834f.

POPOVA, YA. G., ILIEVA, M. AND POPOV, KHR.: (Quantitative method for the determination of riboflavine in culture suspensions and in drug preparations containing tetracyclines and vitamins of the B (B_1, B_2 and PP) group). *Nauch. Tr., Vissh. Inst. Khranit. Vkusova Prom., Plovdiv*, 14, Pt. 1 (1967) 255–259; *C.A.*, 73 (1970) 28959 — TLC and PC.

TELEGDY KOVATS, L., BERNDORFER-KRASZNER, E., PETERFALVI, M. AND GABOR, T.: (Natural occurrence of pangamic acid. I. Pangamic acid content of grains). *Elelmiszervizsgalati Kozlem.*, 15 (1969) 339–348; *C.A.*, 73 (1970) 11222g.

28. ANTIBIOTICS

DZIEGIELEWSKI, K., KOLLER, I., ZUKOWSKI, E., RUCZAJ, D., SMERECZYNSKI, K. AND KARASINSKA, J.: (Effect of packing on viomycin sulphate quality). *Przem. Chem.*, 149 (1970) 161–164; *C.A.*, 73 (1970) 28876w.

LLOYD, P. B. AND CORNFORD, C. C.: A thin-layer chromatographic limit test for the detection of anhydrotetracycline and 4-*epi*-anhydrotetracycline in tetracycline. *J. Chromatogr.*, 53 (1970) 403–405.

RAHMAN, R., SAFE, S. AND TAYLOR, A.: Separation of polythiadioxopiperazine antibiotics by thin-layer chromatography. *J. Chromatogr.*, 53 (1970) 592–594.

See also PC section.

29. INSECTICIDES AND OTHER PESTICIDES

BORS, GH., POPA, I. AND VOICU, A.: (Chromatographic separation of some organochlorine insecticides). *Farmacia (Bucharest)*, 17 (1969) 647–650; *C.A.*, 73 (1970) 13433a.

BURTON, W. B.: Separation of Planavin® herbicide and some related compounds by two-dimensional thin-layer chromatography. *J. Chromatogr.*, 53 (1970) 397–399.

CENCI, P. AND CREMONINI, B.: (Thin-layer chromatography of two organo-tin pesticides and their stability in various soils). *Ind. Sac. Ital.*, 62 (1969) 313–316; *C.A.*, 73 (1970) 13435c.

GEIKE, F.: Dünnschichtchromatographisch-enzymatischer Nachweis von Carbamaten. I. Nachweis insektizider Carbamate mit Rinderleber-Esterase. *J. Chromatogr.*, 53 (1970) 269–277.

KADOUM, A. M.: Thin-layer chromatographic separation and colorimetric detection of malathion and some of its metabolites from stored grains. *J. Agr. Food Chem.*, 18 (1970) 542–543; *C.A.*, 73 (1970) 13436d.

KOUDELA, S.: A modified detection of triazine herbicide residues and their hydroxy-derivatives on thin-layer chromatograms. *J. Chromatogr.*, 53 (1970) 589–591.

SU, Y. C. AND SUN, T. T.: Preparation and purification of aflatoxin. *Ching Kuo Nung Yeh Hua Hsueh Hui Chih*, 7, No. 3–4 (1969) 50–56; *C.A.*, 73 (1970) 33529q.

VEKSHTEIN, M. SH. AND KLISENKO, M. A.: (Identification and quantitative determination of some dialkyldithiocarbamates and their metabolites in different media of vegetable and animal origin). *Vop. Pitan.*, 29, No. 1 (1970) 56–61; *C.A.*, 73 (1970) 13220d.

WANG, R. T. AND WU, F. M.: Simple thin-layer chromatographic method for the detection of pesticide residues in and on vegetables. *T'ai-wan Yao Hsueh Tsa Chih.*, 18, No. 2 (1966) 87–90; *C.A.*, 73 (1970) 13198c.

ZWETKOVA, ZW. M.: Eine schnelle Methode zur Trennung und Identifizierung von chlororganischen, phosphororganischen und Carbamatpesticiden. *Z. Lebensm. Untersuch. Forsch.*, 144 (1970) 268–270.

30. SYNTHETIC AND NATURAL DYES

LEHMANN, G., HAHN, H. G., COLLET, P., SEIFFERT-EISTERT, B. AND MORAN, M.: Beiträge zur Analytik von Farbstoffen. II. Mitt. Schnellmethode zur Bestimmung wasserlöslicher Farbstoffe in Lebensmitteln. *Z. Lebensm. Untersuch. Forsch.*, 143 (1970) 256–263 — preparation of sample.

UNTERHALT, B.: Über die Bestimmung von Lippenstiftfarben. *Z. Lebensm. Untersuch. Forsch.*, 144 (1970) 109–112.

WITTGENSTEIN, E. AND SAWICKI, E.: Analysis of the non-polar fraction of giant ragweed pollen: carotenoids. *Mikrochim. Acta*, (1970) 765–783.

31. PLASTICS AND THEIR INTERMEDIATES

BELENKII, B. G. AND GANKINA, E. S.: Thin-layer chromatography of polymers. Introductory lecture. *J. Chromatogr.*, 53 (1970) 3–25.

MAMENISKIS, W. A.: Determination of poly(oxypropylene)diols present in poly(oxypropylene)-triols by thin-layer chromatography. *J. Appl. Polym. Sci.*, 14 (1970) 1189–1195; *C.A.*, 73 (1970) 26095s.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS

32a. Synthetic drugs and systematic analysis

CONSTANTINESCU, T. AND ENACHE, S.: (Purity of cyclobarbital. I. Chromatographic separations for purity and for determination in unpurified products). *Farmacia (Bucharest)*, 18 (1970) 45–55; *C.A.*, 73 (1970) 28880t.

CONSTANTINESCU, T. AND ENACHE, S.: (Thin-layer chromatographic studies of the purity of phenothiazine). *Arzneimittelstandardisierung*, 19 (1969) 197–202; *C.A.*, 73 (1970) 28881u.

ENACHE, S. AND CONSTANTINESCU, T.: (New efficient possibilities for the analysis of pharmaceutical

preparations by thin-layer separation of active components). *Farmacia (Bucharest)*, 18 (1970) 149–160; *C.A.*, 73 (1970) 38579r.

KELBER, J. J.: (Thin-layer chromatographic separation of some psychotropic molecules). *Bull. Soc. Pharm. Strasbourg*, 13 (1970) 41–45; *C.A.*, 73 (1970) 12634e.

KORCZAK-FABIERKIEWICZ, C. AND CIMBURA, G.: Differentiation of phenothiazine derivatives by locating agent on thin-layer chromatographic plates. *J. Chromatogr.*, 53 (1970) 413–414 — phosphomolybdic acid.

KNIZHNIK, A. Z.: (Use of thin-layer chromatography for the analysis of pharmaceutical preparations prepared from a group of aromatic amines). *Farm. Zh. (Kiev)*, 25, No. 1 (1970) 47–51; *C.A.*, 73 (1970) 28985f.

NAGEL, G.: (Thin-layer chromatographic detection of analgetic agents in suppositories). *Praktikantenbriefe*, 16, No. 2 (1970) 14–16; *C.A.*, 73 (1970) 18510h.

NAITO, S. AND FUKUI, K.: Stability and stabilization of pharmaceuticals. VI. Pharmaceutical studies on 2-aminoethanesulfonic acid derivatives. I. Aminoethylsulfonylphenetidine. I. Stability studies. *Chem. Pharm. Bull.*, 18 (1970) 820–823; *C.A.*, 73 (1970) 28846m.

PAIK, N. H. AND KIM, B. K.: Determination of sulfur-containing organic drugs by means of thin-layer chromatography and the flask combustion method. *Yakhak Hoeji*, 13, No. 2–3 (1969) 84–87; *C.A.*, 73 (1970) 18512k.

PISCHEK, G., KAISER, E. AND KOCH, H.: Die Dünnschichtchromatographie von Thalidomid und seinen Hydrolyseprodukten. *Mikrochim. Acta*, (1970) 530–535.

SKURYDINA, D. F. AND PLESHAKOV, M. G.: (Detection and identification of impurities in 5-ethyl-5-(1-methylbutyl)barbituric acid). *Khim.-Farm. Zh.*, 4, No. 3 (1970) 54–55; *C.A.*, 73 (1970) 18505k.

STEINKE, G. AND SCHMIDT, F.: Dünnschichtchromatographie im Apothekenlaboratorium. 3. Mitt. *Deut. Apotheker- Ztg.*, 110 (1970) 1787–1792.

UNTERHALT, B.: Untersuchungen an neueren Antiphlogistica. *Arch. Pharm.*, 303 (1970) 445–456.

32b. Metabolism of drugs, toxicological applications

DE SILVA, J. A. F. AND PUGLISI, C. V.: Determination of medazepam (Nobrium), diazepam (Valium) and their major biotransformation products in blood and urine by electron capture gas–liquid chromatography. *Anal. Chem.*, 42 (1970) 1725–1736.

KOENIG, J., CHUNDELA, B. AND BAUER, P.: (Preliminary toxicological determination of drugs by thin-layer chromatography; use of prepared plates of Silufol UV_{254}). *Prakt. Lek.*, 50 (1970) 22–23; *C.A.*, 73 (1970) 12732k.

STENLAKE, J. B., WILLIAMS, W. D. AND SKELLERN, G. C.: Development and comparison of thin-layer chromatographic and gas–liquid chromatographic methods for measurement of methimazole in rat urine. *J. Chromatogr.*, 53 (1970) 285–291.

TEWARI, S. N. AND RAM, L.: Separation and identification of parathion and its metabolites in autopsy tissues by thin-layer chromatography. *Mikrochim. Acta*, (1970) 58–60.

32c. Plant extracts

BLAZOVICH, M. AND SPANYAR, P.: (Determination of capsaicin in oleoresin and some other preparations containing large amounts of active principle). *Elelmiszervizsgalati Kozlem.*, 15 (1969) 358–364; *C.A.*, 73 (1970) 13181s.

KURONO, G. AND SASAKI, S.: Quantitative analysis of constituents in crude drugs by thin-layer chromatography. 2. Quantitative analysis of glycyrrhizin in liquorice root by thin-layer chromatography. *Yakugaku Zasshi*, 90 (1970) 497–501; *C.A.*, 73 (1970) 28969d.

33. INORGANIC SUBSTANCES

FREI, R. W. AND STILLMAN, H.: A combination of thin-layer chromatography and emission spectroscopy for the microdetermination of alkaline earth metals. *Mikrochim. Acta*, (1970) 184–189.

GAGLIARDI, E. AND SHAMBRI, H.: Dünnschichtchromatographie von Platinmetallen auf Zellulose. *Mikrochim. Acta*, (1970) 154–163.

GAIBAKYAN, D. S.: (Thin-layer chromatography of rare elements. VI. Identification and separation of rhenium(VII), molybdenum(VI), vanadium(V), and tungsten(VI) in alcoholic and acidified solutions of some ketones. Determination of rhenium). *Arm. Khim. Zh.*, 23, No. 1 (1970) 91–92; *C.A.*, 73 (1970) 31195d.

GAIBAKYAN, D. S.: (Thin-layer chromatography of rare elements. VII. Identification and separation of rhenium(VII), molybdenum(VI), vanadium(V) and tungsten(VI) in ammonia solutions of some alcohols and ketones. Determination of rhenium). *Arm. Khim. Zh.*, 23, No. 1 (1970) 93–94; *C.A.*, 73 (1970) 31199h.

GAIBAKYAN, D. S. AND EGIKYAN, R. T.: (Thin-layer chromatography of rare elements. IX. Identification and determination of gold(III), selenium(IV) and tellurium(IV) in an alkaline medium). *Arm. Khim. Zh.*, 23, No. 1 (1970) 16–21; *C.A.*, 73 (1970) 31258b.

JOHRI, K. N. AND MEHRA, H. C.: Microdetermination of gold(III), silver(I) and ruthenium(III) by ring colorimetry after separation by thin-layer chromatography. *Mikrochim. Acta*, (1970) 807–810.

KASTIEN, H.: Die chromatographische Bestimmung von Blei und Luftstäuben. *Helv. Chim. Acta*, 53 (1970) 2231–2238 — TLC and PC.

MASSA, V.: (Determination of cadmium in the presence of zinc and other metals by dithizonate chromatography and photodensitometry). *Farmaco (Pavia), Ed. Prat.*, 25 (1970) 332–336; *C.A.*, 73 (1970) 38607y.

NAITO, K., TAKEI, S. AND OKABE, T.: Chemical behavior of low-valence sulfur compounds. II. Thin-layer chromatographic separation and photometric determination of low-valence sulfur compounds. *Bull. Chem. Soc. Jap.*, 43 (1970) 1360–1364; *C.A.*, 73 (1970) 41461b.

SMOLYANINOV, V. V.: Electrophoresis and thin-layer chromatography of organic base polythionates. Qualitative analysis. *J. Chromatogr.*, 53 (1970) 337–343 — silica gel with 10% Dowex 50W-X8 and 2% potassium acetate.

VUKCEVIC-KOVACEVIC, V. AND SEREMET, S.: Quantitative analysis of arsenic(III), antimony(III) and bismuth in a mixture, after resolution by thin-layer chromatography. *Bull. Sci. Cons. Acad. Sci. Arts RSF Yougoslavie, Sect. A*, 14 (1969) 377–378; *C.A.*, 73 (1970) 21046s.

See also PC section.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

GAZSO, J. AND BARTHA, K. G.: (Automatic evaluation of the activity distribution on thin-layer plates). *Kiserl. Orvostud.*, 22 (1970) 108–110; *C.A.*, 73 (1970) 21989b.

RAAEN, H. P.: Chromatography of metal ions on thin layers of polytetrafluoroethylene with di-(2-ethylhexyl)orthophosphoric acid. *J. Chromatogr.*, 53 (1970) 605–609 — mobility for 34 cations.

SEILER, H.: Dünnschichtchromatographie als Hilfsmittel in der Radiochemie. 5. Mitt. Abtrennung von Hg aus einem Gemisch von Kationen. *Helv. Chim. Acta*, 53 (1970) 1893–1895.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35a. Surfactants

FAVRETTO, L.: (Characterisation of non-ionic surfactants by means of thin-layer chromatography). *Riv. Ital. Sostanze Grasse*, 47 (1970) 187–191; *C.A.*, 73 (1970) 36843y.

PATTERSON, S. J., SCOTT, C. C. AND TUCKER, K. B. E.: Nonionic detergent degradation. III. Initial mechanism of the degradation. *J. Amer. Oil Chem. Soc.*, 47 (1970) 37–41.

35b. Antioxidants and preservatives

TALUKDAR, P. B. AND DATTA, A. K.: Quantitative determination of paraben in sulfacetamide sodium solution. *Res. Ind.*, 14, No. 2 (1969) 79–81; *C.A.*, 73 (1970) 28974b.

Column Chromatography

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

BEDETTI, G., D'AGNOLO, G. AND POCCHIARI, F.: Anion-exchange chromatography of glycolysis intermediates. *J. Chromatogr.*, 49 (1970) 53–56 — Amberlite AG-IX_4 (Cl^-), Amberlite AG-50WX_4(H^+).

BLASIUS, E. AND KYNAST, G.: Chelate-forming exchange resins. VII. Chelate exchange on naphthalene-1,8-diol-O,O-diacetic acid for the separation of zirconium from hafnium and other elements. *J. Radioanal. Chem.*, 2 (1969) 55–71; *Anal. Abstr.*, 18 (1970) 3071 — the title resin.

ITO, Y. AND BOWMAN, R. L.: Countercurrent chromatography. Liquid–liquid partition chromatography without solid support. *J. Chromatogr. Sci.*, 8 (1970) 315–323 — helix countercurrent chromatography, droplet countercurrent chromatography and locular countercurrent chromatography are described.

JENTOFT, R. E. AND GOUW, T. H.: Pressure-programmed supercritical fluid chromatography of wide molecular weight mixtures. *J. Chromatogr. Sci.*, 8 (1970) 138–142.

KARGER, B. L., CONROE, K. AND ENGELHARDT, H.: The use of surface textured beads for high-speed column liquid chromatography. *J. Chromatogr. Sci.*, 8 (1970) 242–250 — a surface-etched glass bead.

KIRKLAND, J. J. AND DE STEFANO, J. J.: Controlled surface porosity supports with chemically bonded organic stationary phases for gas and liquid chromatography. *J. Chromatogr. Sci.*, 8 (1970) 309–314 — Zipax of controlled surface porosity.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

HRDINA, J.: Sample application to chromatographic columns. *Ger. Pat.*, Offen No. 1, 917, 723; *C.A.*, 72 (1970) 28175w.

HRDINA, J.: Chromatographic analyzer for amino acid mixtures. *Czech. Pat.*, No. 130, 669, 15 Jan. 1969; *C.A.*, 72 (1970) 28249y.

KOCHEN, W. AND LAUER, G.: Simple electronically controlled apparatus for the automation of column chromatography. *Chromatographia*, 2 (1969) 213–214; *Anal. Abstr.*, 19 (1970) 850.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

STRÁNSKÝ, K., STREIBL, M. AND KUBELKA, V.: On natural waxes. XV. Hydrocarbon constituents of the leaf wax from the walnut tree (*Juglans regia* L.). *Coll. Czech. Chem. Commun.*, 35 (1970) 882–891 — SiO_2.

THEILACKER, W. AND KOCH, F.: Über das Bis-(2,5-di-*tert.*-butal-phenyl)methyl. *Chem. Ber.*, 102 (1969) 2020–2025 — Alumina (act. II).

WILLIAMSON, K. L., HSU, Y.-F. L., LACKO, R. AND YOUN, C. H.: The stereochemistry of the Diels–Alder reaction — *syn–anti* Isomerism. *J. Amer. Chem. Soc.*, 91 (1969) 6129–6138 — alumina.

6. ALCOHOLS

NICKON, A., IWADARE, T., MCGUIRE, F. J., MAHAJAN, J. R., NARANG, S. A. AND UMEZAWA, B.: The structure, stereochemistry and genesis of α-caryophyllene alcohol (apollan-11-ol). *J. Amer. Chem. Soc.*, 92 (1970) 1688–1696 — alumina.

10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

ČAPEK, K., ČAPKOVÁ-ŠTEFFKOVÁ, J. AND JARÝ, J.: Partial acetylation and deacetylation of derivatives of methyl 3,6-dideoxy-α-D-xylo-hexopyranoside. *Coll. Czech. Chem. Commun.*, 35 (1970) 321–326 — silica gel.

ČAPEK, K. AND JARÝ, J.: Preparation of methyl 2,3-anhydro-4,6-dideoxy-α,D-lyxo- and ribo-hexopyranoside. *Coll. Czech. Chem. Commun.*, 35 (1970) 1727–1732 — alumina, silica gel.

ČAPEK, K., KEFURTOVÁ, Z. AND JARÝ, J.: Amino sugars. XXIII. A contribution to the aminolysis of methyl 2,3-anhydro-4,6-O-benzylidene-α,D-allopyranoside. *Coll. Czech. Chem. Commun.*, 35 (1970) 1930–1931 — silica gel.

TRÉNEL, G. AND EMEIS, C. C.: Analyse und Charakterisierung von Glucosesirup mit Hilfe der Gelchromatographie. *Stärke*, 22 (1970) 188–191.

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

BERST, M., HELLERQUIST, C. G., LINDBERG, B., LÜDERITZ, O., SVENSSON, S. AND WESTIMAL, O.: Structural investigations on T1 lipopolysaccharides. *Eur. J. Biochem.*, 11 (1969) 353–359 — Sephadex G-200.

SILBERT, J. E. AND DELUCA, S.: Degradation of glycosaminoglycans by tadpole tissue. Differences in activity toward chondroitin 4-sulfate and chondroitin 6-sulfate. *J. Biol. Chem.*, 245 (1970) 1506–1508 — Sephadex G-100, G-200.

13. STEROIDS

DOMB, S. AND SCHAFFNER, K.: Photochemical reactions. Part 58. On the mechanism of the rearrangement of the triplet-excited α,β-unsaturated δ-diketone 3,7-dioxo-4,4-dimethyl-17β-acetoxy-Δ^5-androstene. *Helv. Chim. Acta*, 53 (1970) 677–684 — silica gel.

SUCROW, W. AND GIRGENSOHN, B.: Die Synthese von 5α-Stigmastadien-(7,25)-ol-(3β). *Chem. Ber.*, 103 (1970) 745–749 — alumina.

14. STEROID GLYCOSIDES AND SAPONINS

SANER, A., ZERLENTIS, C., STÖCKLIN, W. AND REICHSTEIN, T.: Glykoside und Aglykone. 325. Mitt. Die Glykoside von *Marsdenia erecta* R. Br. 1. Isolierungen. *Helv. Chim. Acta*, 53 (1970) 221–245 — Silica gel, alumina.

SIERP, D., STÖCKLIN, W. AND REICHSTEIN, T.: Glykoside und Aglykone. 323. Mitt. Inhaltstoffe der Wurzeln von *Margaretta rosea* Oliv. *Helv. Chim. Acta*, 53 (1970) 27–47 — silica gel.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

BÜCHI, G., HOFHEINZ, W. AND PAUKSTELIS, J. V.: The synthesis of (–)-Aromadendrene and related sesquiterpenes. *J. Amer. Chem. Soc.*, 9 (1969) 6473–6478 — Alumina.

DEMOLE, E., ENGGIST, P., SÄUBERLI, U. AND STOLL, M.: Structure et synthèse de la damascénone (triméthyl-2,6,6-*trans*-crotonoyl-1-cyclo-hexadiène-1,3), constituant odorant de l'essence de rose bulgare (*Rosa damascena* Mill.). *Helv. Chim. Acta*, 53 (1970) 541–551 — Silicic acid.

HUBER, U. A. AND DREIDING, A. S.: Synthese von terpenartigen bicyclischen Systemen. Über die Cycloaddition von Dimethyl-keten an Methylcyclopentadien. *Helv. Chim. Acta*, 53 (1970) 495–513 — Kieselgel.

OHLOFF, G., STRICKLER, H., WILLHALM, B., BORER, C. AND HINDER, M.: Über En-Synthesen mit Singulett-Sauerstoff. II. Die Farbstoff-sensibilisierte Photooxygenierung von (–)-Thujopsen und die Stereochemie der dargestellten Thujopsanole. *Helv. Chim. Acta*, 53 (1970) 623–637 — silica gel.

THOMAS, A. F. AND BUCHER, W.: Menthatrienes and the oxidation of limonene. *Helv. Chim. Acta*, 53 (1970) 770–775 — silica gel.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

AGRAWAL, B. B. L. AND MARGOLIASM, E.: A spectrophotometric method for the determination of aminotriazole and other aromatic amines. *Anal. Biochem.*, 34 (1970) 505–516 — Sephadex G-15.

ROSENMUND, P., SAUER, D. AND TROMMER, W.: Beiträge zur Chemie des Indols. II. Beckmann- und Schmidt-Umlagerungen an einigen Indolketonen. *Chem. Ber.*, 103 (1970) 496–509 — Kieselgel.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19b. Elucidation of structure of proteins

LEE, N., PATRICK, J. W. AND BARNES, N. B.: Subunit structure of L-ribulokinase from *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1357–1361 — Sephadex G-25.

NEER, E. J.: The reactions of the sulfhydryl groups of human hemoglobin β_4. *J. Biol. Chem.*, 245 (1970) 564–569 — Dowex 50 X2, Sephadex G-50.

RIGOPOULOU, D., VALVERDE, I., MARCO, J., FALCONA, G. AND UNGER, R. H.: Large glucagon immunoreactivity in extracts of pancreas. *J. Biol. Chem.*, 245 (1970) 496–501 — Bio-gel P-10.

SCHOENMAKERS, J. G. G., GERDING, J. J. T. AND BLOEMENDAL, H.: The subunit structure of α-crystallin. Isolation and characterization of the S-carboxymethylated acidic subunits from adult and embryonic origin. *Eur. J. Biochem.*, 11 (1969) 472–481 — Sephadex G-200, Dowex 50W X2, SE-Sephadex C-50.

20. PROTEINS (INCLUDING ENZYMES)

20a. Proteins of plant origin including bacteria

REINHAMMAR, B.: Purification and properties of laccase and stellacyanin from *Rhus vernicifera*. *Biochim. Biophys. Acta*, 205 (1970) 35–47 — DEAE-Sephadex A-50, CM-Sephadex C-50.

SANDULESCU, C.: (Quantitative Veränderungen der Eiweissfraktionen des Getreidemehles während der Reifung. Vorläufige Ergebnisse). *Ind. Aliment. (Bucharest)*, 21 (1970) 194–196.

SANDULESCU, C.: (Gelchromatographie der Eiweisstoffe des Klebers und Aminosäurezusammensetzung). *Ind. Aliment. (Bucharest)*, 21 (1970) 141–144.

20b. Plasma proteins

DE URIST, G. M., LANCKMAN, M. AND HAMERS, R.: Separation of two types of light polypeptide chains from rabbit γ-globulins. *Eur. J. Biochem.*, 11 (1969) 370–375 — DEAE-cellulose, Sephadex G-100.

FORRESTER, J. A., ARNOLD, R. AND RUHENSTROTH-BAUER, G.: Isolation of an ionic strength dependent cell agglutinin from bovine seminal plasma. *Eur. J. Biochem.*, 11 (1969) 341–346 — Sephadex G-100, carboxymethyl Sephadex C-25.

FRANĚK, F. AND NOVOTNÝ, J.: Isolation of a disulfide bridge containing fragment of the variable part of pig immunoglobulin λ-chains. *Eur. J. Biochem.*, 11 (1969) 165–169 — Sephadex G-100, SE-Sephadex.

HESS, M. AND HILSCHMANN, N.: Genetischer Polymorphismus im konstanten Teil von humanen Immunoglobulin-L-Ketten vom λ-Typ. *Z. Physiol. Chem.*, 351 (1970) 67–73 — Dowex 1 X2, 50W X2.

20c. Structural proteins

KEFALIDES, N. A. AND FORSELL-KNOTT, L.: Structural changes in the protein and carbohydrate components of glomerular basement membrane in aminonucleoside nephrosis. *Biochim. Biophys. Acta*, 203 (1970) 62–66 — Sephadex G-10, Dowex 50 X8.

20d. Protamines, histones and other nuclear proteins

RALL, S. C. AND COLE R. D.: Quantitative cleavage of a protein with N-bromosuccinimide. *J. Amer. Chem. Soc.*, 92 (1970) 1800–1801 — Sephadex G-150.

STOLLAR, B. D. AND WARD, M.: Rabbit antibodies to histone fractions as specific reagents for preparative and comparative studies. *J. Biol. Chem.*, 245 (1970) 1261–1266 — CM-cellulose.

20f. Varia, with special reference to non-identified and tissue proteins

LUKENS, L. N.: The formation of collagen α chains in the absence of proline hydroxylation. *J. Biol. Chem.*, 245 (1970) 453–461 — CM-cellulose, Sephadex G-25, G-200.

SCHIFFER, M. ,HARDMAN, K. D., WOOD, M. K., EDMUNDSON, A. B., HOOK, M. E., ELY, K. R. AND DEUTSCH, H. F.: A preliminary crystallographic investigation of a human L-type Bence–Jones protein. *J. Biol. Chem.*, 245 (1970) 728–730 — Sephadex G-100.

20g. Enzymes: oxidoreductases

BABIOR, B. M.: The mechanism of action of ethanolamine deaminase. VI. Ethylene glycol, a quasisubstrate for ethanolamine deaminase. *J. Biol. Chem.*, 245 (1970) 1755–1766 — Dowex 1 X8, 50 X2, Bio-gel P-2.

BANNERJEE, D., SANDERS, L. E. AND SOKATCH, J. R.: Properties of purified methylmalonate semialdehyde dehydrogenase of *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 245 (1970) 1828–1835 — DEAE-cellulose, Sephadex G-200.

COLMAN, R. F. AND CHU, R.: The role of sulfhydryl groups in the catalytic function of isocitrate dehydrogenase. III. Effect of N-ethylmaleimide on chemical and physical properties. *J. Biol. Chem.*, 245 (1970) 608–615 — Sephadex G-150.

DUCKWORTH, H. W. AND COLEMAN, J. E.: Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.*, 245 (1970) 1613–1625 — DEAE-cellulose.

ENGLARD, S., KAYSEN, G. AND AVIGAD, G.: 5-Keto-D-fructose. VI. A specific reduced nicotinamide adenine dinucleotide phosphate-linked reductase from yeast. *J. Biol. Chem.*, 245 (1970) 1311–1318 — Sephadex G-100, DEAE-Sephadex.

FUJIOKA, S. AND SILBER, R.: Purification and properties of ribonucleotide reductase from leukemic mouse spleen. *J. Biol. Chem.*, 245 (1970) 1688–1693 — Sephadex G-25.

GEORGE, D. J. AND PHILLIPS, A. T.: Identification of α-ketobutyrate as the prosthetic group of urocanase from *Pseudomonas putida*. *J. Biol. Chem.*, 245 (1970) 528–537 — DEAE-cellulose, Sephadex G-200, G-25.

HEINZ, F. AND KULBE, K. D.: Glyceraldehyde phosphate dehydrogenase from liver. I. Isolation and characterization of the bovine liver enzyme. *Z. Physiol. Chem.*, 351 (1970) 249–262 — DEAE-cellulose, Sephadex G-75, G-150.

HOLLOWAY, P. W. AND WAKIL, S. J.: Requirement for reduced diphosphopyridine nucleotide-cytochrome b_5 reductase in stearyl coenzyme A desaturation. *J. Biol. Chem.*, 245 (1970) 1862–1865 — DEAE-cellulose.

ICHIYAMA, A., NAKAMURA, S., NISHIZUKA, Y. AND HAYAISHI, O.: Enzymic studies on the biosynthesis of serotonin in mammalian brain. *J. Biol. Chem.*, 245 (1970) 1699–1709 — Sephadex G-25, Amberlite CG-50, Dowex 1.

KIRTLEY, M. E. AND KOSHLAND, JR., D. E.: The properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase labeled with a "reporter group". *J. Biol. Chem.*, 245 (1970) 276–286 — Sephadex G-25.

20h. Enzymes; transferases

DER TERROSSIAN, E., PRADEL, L. A., KASSAB, R. AND THOAI, N. V.: Comparative structural studies of the active site of ATP-guanidine phosphotransferases. The essential cysteine tryptic peptide of arginine kinase from *Homarus vulgaris* muscle. *Eur. J. Biochem.*, 11 (1969) 482–490 — Sephadex G-25, G-15.

GREENE, R. AND KORN, D.: Partial purification and characterization of deoxyribonucleic acid polymerase from KB cells. *J. Biol. Chem.*, 245 (1970) 254–261 — phosphocellulose.

HENDERSON, E. J., ZALKIN, H. AND HWANG, L. H.: The anthranilate synthetase–anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase aggregate. Catalytic and regulatory properties of aggregated and unaggregated forms of anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase. *J. Biol. Chem.*, 245 (1970) 1424–1431 — DEAE-cellulose.

JERGIL, B. AND DIXON, G. H.: Protamine kinase from rainbow trout testis. Partial purification and characterization. *J. Biol. Chem.*, 245 (1970) 425–434 — Sephadex G-10, G-200, DEAE-cellulose.

KIM, S. AND PAIK, W. K.: Purification and properties of protein methylase. II. *J. Biol. Chem.*, 245 (1970) 1806–1813 — Sephadex G-100, DEAE-Sephadex A-50.

KOBATA, A. AND GINSBURG, V.: Uridine diphosphate-N-acetyl-D-galactosamine: D-galactose α-3-N-acetyl-D-galactosaminyltransferase, a product of the gene that determines blood type A in man. *J. Biol. Chem.*, 245 (1970) 1484–1490 — Sephadex G-200.

PAYNE, K. J. AND BOEZI, J. A.: The purification and characterization of adenosine triphosphate-ribonucleic acid adenylyltransferase from *Pseudomonas putida*. *J. Biol. Chem.*, 245 (1970) 1378–1387 — Sephadex G-100, DEAE-Sephadex, cellulose phosphate.

WHITFIELD, C. D., STEERS, JR., E. J. AND WEISSBACH, H.: Purification and properties of 5-methyl-tetrahydropteroyltriglutamate-homocysteine transmethylase. *J. Biol. Chem.* 245 (1970) 390–401 — DEAE-Sephadex, hydroxylapatite, Sephadex G-100.

WHITFIELD, C. D. AND WEISSBACH, H.: Binding of the folate substrate to 5-methyl-tetrahydropteroyltriglutamate-homocysteine transmethylase. *J. Biol. Chem.*, 245 (1970) 402–409 — Sephadex G-50.

WILCHEK, M. AND GORECKI, M.: Affinity chromatography of bovine pancreatic ribonuclease A. *Eur. J. Biochem.*, 11 (1969) 491–494 — Sepharose 4 B, Dowex 50X8.

20i. Enzymes; hydrolases

AMINOFF, D. AND FURUKAWA, K.: Enzymes that destroy blood group specificity. I. Purification and properties of α-L-fucosidase from *Clostridium perfringens*. *J. Biol. Chem.*, 245 (1970) 1659–1669 — Sephadex G-75, G-200, alumina, Celite.

BAHL, O. P.: Glycosidases of *Aspergillus niger*. II. Purification and general properties of 1,2-α-L-fucosidase. *J. Biol. Chem.*, 245 (1970) 299–304 — DEAE-Sephadex A-50, Sephadex G-150.

GRIFFIN, T. B. AND PRESCOTT, J. M.: Some physical characteristics of a proteinase from *Aeromonas proteolytica*. *J. Biol. Chem.*, 245 (1970) 1348–1356 — DEAE-Sephadex A-50, Sephadex G-75, G-200.

KLEE, W. A. AND STREATY, R. A.: Studies on the conformation and proteolytic susceptibility of rat and bovine pancreatic ribonucleases. *J. Biol. Chem.*, 245 (1970) 1227–1232 — Amberlite IRC-50, SE-Sephadex C-25, CM-cellulose.

NUMMI, M., DAUSSANT, J., NIKU-PAAVOLA, M., KALSTA, H. AND ENARI, T. M.: Comparative immunological and chromatographic study of some plant β-amylases. *J. Sci. Food Agr.*, 21 (1970) 258–260.

20k. Enzymes; lyases

ANDERSON, P. J., GIBBONS, I. AND PERHAM, R. N.: A comparative study of the structure of muscle fructose 1,6-diphosphate aldolases. *Eur. J. Biochem.*, 11 (1969) 503–509 — Sephadex G-75.

HATFIELD, G. W., RAY, JR., W. J. AND UMBARGER, H. E.: Threonine deaminase from *Bacillus subtilis*. III. Pre-steady state kinetic properties. *J. Biol. Chem.*, 245 (1970) 1748–1754 — Sephadex G-25.

HATFIELD, G. W. AND UMBARGER, H. E.: Threonine deaminase from *Bacillus subtilis*. I. Purification of the enzyme. *J. Biol. Chem.*, 245 (1970) 1736–1741 — ECTEOLA-cellulose.

HOU, C. T. AND PERLMAN, D.: Microbial transformations of peptide antibiotics. V. Purification and properties of the actinomycin lactonase from *Actinoplanes missouriensis*. *J. Biol. Chem.*, 245 (1970) 1289–1295 — calcium phosphate cellulose, DEAE-cellulose, Sephadex G-200.

KUMAGAI, H., YAMADA, H., MATSUI, H., OHKISHI, H. AND OGATA, K.: Tyrosine phenol lyase. I. Purification, crystallization and properties. *J. Biol. Chem.*, 245 (1970) 1767–1772 — DEAE-Sephadex A-50, Sephadex G-150, hydroxylapatite.

KUMAGAI, H., YAMADA, H., MATSUI, H., OHKISHI, H. AND OGATA, K.: Tyrosine phenol lyase. II. Cofactor requirements. *J. Biol. Chem.*, 245 (1970) 1773–1777 — Sephadex G-25.

SHEDLARSKI, J. G. AND GILVARG, C.: The pyruvate-aspartic semialdehyde condensing enzyme of *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1362–1373 — hydroxylapatite, Sephadex G-25.

20m. Enzymes; ligases

DE LORENZO, F. AND AMES, B. N.: Histidine regulation in *Salmonella typhimurium*. VII. Purification and general properties of the histidyl transfer ribonucleic acid synthetase. *J. Biol. Chem.*, 245 (1970) 1710–1716 — Sephadex G-25, hydroxylapatite, DEAE-Sephadex, phosphocellulose.

HAYASHI, H., KNOWLES, J. R., KATZE, J. R., LAPOINTE, J. AND SÖLL, D.: Purification of leucyl transfer ribonucleic acid synthetase from *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1401–1406 — DEAE-cellulose, hydroxylapatite.

KNOWLES, J. R., KATZE, J. R., KONIGSBERG, W. AND SÖLL, D.: The interaction of seryl and of leucyl transfer ribonucleic acid synthetases with their cognate transfer ribonucleic acids. *J. Biol. Chem.*, 245 (1970) 1407–1415 — Bio-gel P-150, Sephadex G-200.

PAPAS, T. S. AND MEHLER, A. H.: Analysis of amino acid binding to the proline transfer ribonucleic acid synthetase of *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1588–1595 — hydroxylapatite, DEAE-cellulose.

SAMUEL, D., ESTROUMZA, J. AND AILHAUD, G.: Partial purification and properties of acylCoA synthetase of *Escherichia coli*. *Eur. J. Biochem.*, 12 (1970) 576–582 — DEAE-cellulose, hydroxylapatite, Dowex 1 X 8, Sephadex G-200.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

JOST, J.-P., HSIE, A., HUGHES, S. D. AND RYAN, L.: Role of cyclic adenosine 3′,5′-monophosphate in the induction of hepatic enzymes. I. Kinetics of the induction of rat liver serine dehydratase by cyclic adenosine 3′,5′-monophosphate. *J. Biol. Chem.*, 245 (1970) 351–357 — Dowex AG1 X2.

PILLARISETTY, R. J. AND KARASEK, M. A.: Pyrimidine biosynthesis *de novo* in skin. *J. Biol. Chem.*, 245 (1970) 358–362 — Dowex 1 X8, 50 X8.

SHIMA, T., FUJIMURA, S., HASEGAWA, S., SHIMIZU, Y. AND AND SUGIMURA, T.: Studies of poly adenosine diphosphate-ribose. VIII. A method for purification of poly adenosine diphosphate-ribose. *J. Biol. Chem.*, 245 (1970) 1327–1330 — Sephadex G-50.

21b. Nucleic acids: RNA

CALDWELL, I. C. AND HENDERSON, J. F.: Isolation of nucleotides, nucleic acids and protein from single tissue samples by a phenol technique. *Anal. Biochem.*, 34 (1970) 303–311 — Sephadex G-100, Bio-gel P-2.

CHIRIKDJIAN, J. G. AND DAVIS, F. F.: Compositional variations in the common pentanucleotide from transfer ribonucleic acids of *Escherichia coli*. *J. Biol. Chem.*, 245 (1970) 1296–1301 — DEAE-cellulose.

ERHARDT, F. AND ZACHAU, H. G.: Reversible Inaktivierung von Transfer-Ribonucleinsäuren mit Diketen. *Z. Physiol. Chem.*, 351 (1970) 567–570 — Sephadex G-100.

HIRSCH, R. AND ZACHAU, H. G.: Zur Modifizierung spezifischer Transfer-Ribonucleinsäure durch Jod. *Z. Physiol. Chem.*, 351 (1970) 563–566 — DEAE-cellulose.

PHILIPPS, G. R.: Binding of transfer ribonucleic acid to ribosome. Comparison of the nonenzymatic binding of aminoacylated and deacylated transfer ribonucleic acid. *J. Biol. Chem.*, 245 (1970) 859–868 — Sephadex G-100.

ROY, K. L. AND SÖLL, D.: Purification of five serine transfer ribonucleic acid species from *Escherichia coli* and their acylation by homologous and heterologous seryl transfer ribonucleic acid synthetases. *J. Biol. Chem.*, 245 (1970) 1394–1400 — DEAE-cellulose, DEAE-Sephadex A-50.

WILCOX, M.: γ-Glutamyl phosphate attached to glutamine-specific tRNA. A precursor of glutaminyl tRNA in *Bacillus subtilis*. *Eur. J. Biochem.*, 11 (1969) 405–412 — Sephadex G-100, Dowex 50, Bio-gel A, DEAE-cellulose.

21c. Nucleic acids: DNA

FUKUHARA, H.: Relative proportions of mitochondrial and nuclear DNA in yeast under various conditions of growth. *Eur. J. Biochem.*, 11 (1969) 135–139 — hydroxylapatite.

PROBST, H.: Elution neusynthetisierter DNA aus Säugetier-Zellen von Methylalbumin-Kieselguhr-Säulen durch Guinidinthiocyanat. *Z. Physiol. Chem.*, 351 (1970) 163–168 — MAK.

22. ALKALOIDS

KUTNEY, J. P., ABDURAHMAN, N., GLETSOS, C., LE QUESNE, P., PIERS, E. AND VLATTAS, I.: Total synthesis of indole and dihydroindole alkaloids. V. The total synthesis of *dl*-quebrachamine and *dl*-aspidospermidine. A general entry into the aspidosperma alkaloids. *J. Amer. Chem. Soc.*, 92 (1970) 1727–1735 — alumina.

KUTNEY, J. P., BROWN, R. T., PIERS, E. AND HADFIELD, J. R.: Total synthesis of indole and dihydroindole alkaloids. III. The transannular cyclization of carbomethoxydihydro-cleavamine and carbomethoxycleavamine derivatives. An approach to Vinca and Iboga alkaloids. *J. Amer. Chem. Soc.*, 92 (1970) 1708–1712 — alumina.

KUTNEY, J. P., CRETNEY, W. J., LE QUESNE, P., MCKAGUE, B. AND PIERS, E.: Total synthesis of indole and dihydroindole alkaloids. IV. The total synthesis of *dl*-dihydrocleavamine, *dl*-carbomethoxydihydrocleavamine, *dl*-coronaridine, *dl*-dihydrocatharanthine, and *dl*-ibogamine. A general entry into the Iboga and Vinca alkaloids. *J. Amer. Chem. Soc.*, 92 (1970) 1712–1726 — alumina.

NARANJO, J., HESSE, M. AND Schmid, H.: Alkaloide. 137. Mitt. Über das Alkaloid Lonicerin aus *Callichilia barteri*. *Helv. Chim. Acta*, 53 (1970) 749–754 — Kieselgel.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

SMITH, H. W. AND RAPOPORT, H.: Mechanism of the transformation of cyclopenin to viridicatin. *J. Amer. Chem. Soc.*, 91 (1969) 6083–6089 — silica gel.

24. ORGANIC SULPHUR COMPOUNDS

BOHLMANN, F. AND ZDERO, CH.: Polyacetylenverbindungen. 173. Über die Inhaltsstoffe aus *Eclipta erecta* L. *Chem. Ber.*, 103 (1970) 834-841 — alumina (act. II).

RABAN, M., KENNEY, Jr., G. W. J. AND JONES, Jr., F. B.: Stereochemistry at trivalent nitrogen. VI. Slow rotation about nitrogen–sulfur formal single bonds in N,N-dialkylsulfenamides. *J. Amer. Chem. Soc.*, 91 (1969) 6677–6683 — silica gel.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

BLACKMER, G. L., HAMM, R. E. AND LEGG, J. I.: Synthesis and characterization of some cobalt (III) complexes of ethylenediaminetriacetic acid. *J. Amer. Chem. Soc.*, 91 (1969) 6632–6637 — Dowex 1 X8, 50W X8.

Gas Chromatography

1. REVIEWS AND BOOKS

CURRY, A.: *Poison Detection in Human Organs*, 2nd ed. Springfield, Ill., 1969, 280 + XXIV pp. — GC included.

GIRY, L.: Applications récentes de la spectrométrie de masse associée à d'autres techniques. *Chim. Anal. (Paris)*, 52 (1970) 1089–1100.

2. FUNDAMENTALS, THEORY AND GENERAL

2d. General

SHEVCHUK, I. M., BOGOSLOVSKIĬ, YU. N. AND SAKHAROV, V. M.: (The relationship between chromatographic and thermodynamic characteristics of hydrocarbons.) *Neftekhimiya*, 10 (1970) 913–916.

3. TECHNIQUES I

3a. Detectors

CRAVEN, D. A.: Simplified version of the alkali flame detector for nitrogen mode operation. *Anal. Chem.*, 42 (1970) 1679–1680.

DAGNALL, R. M., PRATT, S. J., WEST, T. S. AND DEANS, D. R.: A microwave-excited emissive detector for gas chromatography. Further studies with sulphur compounds. *Talanta*, 17 (1970) 1009–1013.

DEVAUX, P. AND GUIOCHON, G.: Determination of the optimum operating conditions of the electron capture detector. *J. Chromatogr. Sci.*, 8 (1970) 502–508.

FENIMORE, D. C. AND DAVIS, C. M.: Linear electron capture detection. *J. Chromatogr. Sci.*, 8 (1970) 519–523.

KIENITZ, H.: Massenspektrometrie unter den Bedingungen der Kopplung mit der Gas-Chromatographie. *Z. Anal. Chem.*, 252 (1970) 350–358.

SOKOLOV, D. N. AND VAKIN, N. A.: (Superhigh temperature diaphragm detector for gas chromatography). *Zavodsk. Lab.*, 36 (1970) 1314–1315.

WINNETT, G.: A simplified reignition system for flame photometric detectors. *J. Chromatogr. Sci.*, 8 (1970) 554–554.

3b. Column performance and filling studies

ANNINO, R. AND MCCREA, P. F.: Variable selectivity stationary phases for gas chromatography. *Anal. Chem.*, 42 (1970) 1486–1491.

DI CORCIA, A., FRITZ, D. AND BRUNER, F.: Use of graphitized carbon black for linear gas–liquid–solid chromatography of polar low-boiling compounds. *Anal. Chem.*, 42 (1970) 1500–1504.

HRIVŇÁK, J.: Use of phosphoric acid additive for gas chromatographic analysis on open tubular columns. *J. Chromatogr. Sci.*, 8 (1970) 602–603.
POPOVA, A. M., KAREL'SKAYA, V. F. AND PLACHENOV, T. G.: (Study of the influence of the porous structure of carbon adsorbents on their gas chromatographic characteristics). *Zh. Prikl. Khim.*, 43 (1970) 2227–2230.
WALKER, J. Q. AND WOLF, C. J.: Advantages of a dual column (packed and capillary columns in series) for gas chromatographic analysis. *Anal. Chem.*, 42 (1970) 1652–1654.

3c. Apparatus, accessories and materials for GC

AVERETT, W. R.: Corrosive gas standards for gas chromatography. *J. Chromatogr. Sci.*, 8 (1970) 552–552.
GOLTZ, H. L. AND MOFFAT, J. B.: An apparatus and technique for sampling from a vacuum system to a gas chromatograph. *J. Chromatogr. Sci.*, 8 (1970) 596–599.
GUGLYA, V. G. AND IVANOV, G. A.: (Determination of chromatographic characteristics of stationary phases by means of piezoquartz microweighing.) *Zh. Anal. Khim.*, 25 (1970) 2224–2247.
GÜNTHER, H.: Kopplung von Gas-Chromatographie und Infrarotspektroskopie mit dem Extrocell-System. *Z. Anal. Chem.*, 252 (1970) 145–146.
HORTON, A. D. AND LINGINFELTER, H. G.: Prevention of backlash of the recorder pen on the Aerograph 713 preparative gas chromatograph. *J. Chromatogr. Sci.*, 8 (1970) 539–539.
KAISER, R.: Probleme der Kopplung Gas-Chromatographie mit Massenspektrometrie im Bereich niederer Konzentrationen. *Z. Anal. Chem.*, 252 (1970) 119–123.
KIENITZ, H.: Massenspektrometrie unter den Bedingungen der Kopplung mit gas-chromatographischen Trennverfahren. *Z. Anal. Chem.*, 252 (1970) 118–118.
KISHIMOTO, K. AND KINOSHITA, K.: (A factor of ghost and memory peaks and improvement of injection system in gas chromatography). *Japan Analyst*, 18 (1969) 588–592.
LEVY, R. L. WOLF, C. J. AND ORÓ, J.: A gas chromatographic method for characterization of the organic content present in an inorganic matrix. *J. Chromatogr. Sci.*, 8 (1970) 524–526.
MATHEWS, R. G., SCHWARTZ, R. D., STOUFFER, J. E. AND PETTITT, B. C.: New polyamide liquid phases for gas chromatography. *J. Chromatogr. Sci.*, 8 (1970) 508–512.
WALKER, J. Q. AND WOLF, C. J.: Pyrolysis gas chromatography: A comparative study of different pyrolyzers. *J. Chromatogr. Sci.*, 8 (1970) 513–518.

4. TECHNIQUES II

4a. Preparative-scale GC

ALBRECHT, J. AND VERZELE, M.: Preparative scale gas chromatography. X. Improved packing method for large diameter columns. *J. Chromatogr. Sci.*, 8 (1970) 586–591.
CRAVEN, D. A.: Optimization of production-scale GLC. *J. Chromatogr. Sci.*, 8 (1970) 540–543.

4b. Programmed temperature and programmed pressure GC

BEREZKIN, V. G., TATARINSKIĬ, V. S. AND ZHUKHOVITSKIĬ, A. A.: (A new modification of chromathermography). *Zavodsk. Lab.*, 36 (1970) 1299–1301.
YUSFIN, V. S., GRIGOR'EV, G. A., DATSKEVICH, A. A. AND ZHUKHOVITSKIĬ, A. A.: (Adsorption concentration on the basis of the combination of thermal effect with programming of velocity). *Zavodsk. Lab.*, 36 (1970) 1297–1299.

4d. Special microtechniques and functional analysis

GOUVERNEUR, P.: Recent advances in organic elemental analysis. *Chem. Ind. (London)*, (1970) 1464–1465.
IGNASIAK, B. S., NANDI, B. N. AND MONTGOMERY, D. S.: Direct determination of oxygen in coal. *Anal. Chem.*, 41 (1969) 1676–1678.
KELLEY, J. D. AND WOLF, C. J.: A comparison of pyrolysis gas chromatography and mass spectrometry. *J. Chromatogr. Sci.*, 8 (1970) 583–585.
MACGEE, J. AND ALLEN, K. G.: Alkylation and gas chromatography of aqueous inorganic halides. *Anal. Chem.*, 42 (1970) 1672–1673.
RÜSSEL, H. A.: Gas-chromatographische Fluorbestimmung in Organmaterial und Körperflüssigkeiten. *Z. Anal. Chem.*, 252 (1970) 143–145.

4e. Automation

BAUDISCH, J. AND PAPENDICK, H. D.: Konzeption eines zentralen GC-Datenverarbeitungssystems. *Erdoel Kohle*, 23 (1970) 707–707.

4f. Measurement of physico-chemical and related values

ABDEDDAÏM, K. AND VERGNAUD, J.-M.: Théorie simplifiée de la chromatographie avec variation de débit et de la chromatographie avec inversion du sens du gaz vecteur (backflushing). *Chim. Anal. (Paris)*, 52 (1970) 1103–1108.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

POZDNEV, V. V., PARFENOV, A. I., BABINA, YU. K. AND SHKOLINA, L. A.: (Analysis of acetylene from carbide by gas chromatography). *Zavodsk. Lab.*, 36 (1970) 1310–1312.

5b. Other hydrocarbons

DZHIOEVA, Z. K., ALFEROVA, V. V., SAVEL'EVA, I. P. AND SKIBINSKAYA, M. B.: (Gas chromatographic analysis of the products from the synthesis of hexachlorocyclopentadiene). *Zavodsk. Lab.*, 36 (1970) 1326–1327.

KRYUKOV, S. I., KUT'IN, A. M., VERNOVA, T. P., ERATOVA, V. L. AND PREOBRAZHENSKIĬ, N. A.: (Technical synthesis of methylpentadiene). *Khim. Prom.*, 45 (1969) 256–258.

SCHLOTZHAUER, W. S., SCHMELTZ, I. AND OSMAN, S. F.: Evidence for the origin of monoalkenes in cigarette smoke. *Chem. Ind., (London)*, (1970) 1377–1378.

WASIK, S. P. AND TSANG, W.: Gas–liquid chromatography separations of hydrocarbons using columns with aqueous solutions of complexing metal-ions as stationary phases. *Anal. Chem.*, 42 (1970) 1648–1649.

7. PHENOLS

KUSHNIR, I., BARR, P. A. AND CHORTYK, O. T.: An improved quantitative method for volatile phenols. *Anal. Chem.*, 42 (1970) 1619–1621.

MOSKOVSKIKH, V. V., KHARLAMPOVICH, G. D. AND ISAENKO, L. A.: (Study of stationary liquid phases applied in chromatographic analysis of phenols). *Zh. Fiz. Khim.*, 44 (1970) 2891–2895 — very limited literature search on the topic.

9. OXO COMPOUNDS

GLOWACKI, C. R., MENARDI, P. J. AND LINK, W. E.: The analysis of 1,2-epoxyalkanes by gas–liquid chromatography. *J. Amer. Oil Chem. Soc.*, 47 (1970) 225–228.

10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

PARTRIDGE, R. D. AND WEISS, A. H.: Extraction procedure for TMS carbohydrate ethers. *J. Chromatogr. Sci.*, 8 (1970) 553–553.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

DENCKER, W. D. AND WOLF, C. J.: Vapor phase pyrolysis gas chromatography of methyl esters. *J. Chromatogr. Sci.*, 8 (1970) 534–539.

HEJGAARD, J.: Determination of α-acyl-α-hydroxy acids in biological fluids by head-space gas chromatography. *Anal. Biochem.*, 37 (1970) 368–377.

MORII, T. AND ARIMOTO, H.: (Adsorption gas chromatography of organic acids with steam-containing carrier gas). *Japan Analyst*, 18 (1969) 900–903.

11b Lipids and their constituents

CHRISTIE, W. W., NOBLE, R. C. AND MOORE, J. H.: Determination of lipid classes by a gas chromatographic procedure. *Analyst*, 95 (1970) 940–944.

CHRISTOPHE, A.: Regeneration of an analytical column for fatty acid methyl ester analysis. *J. Chromatogr. Sci.*, 8 (1970) 614–615.

EGGE, H., MURAWSKI, U., RYHAGE, R., ZILLIKEN, F. AND CYOERGY, P.: Positional isomers of monoenoic fatty acids from human milk. *Z. Anal. Chem.*, 252 (1970) 123–126.

HÖLZL, J.: Radiogaschromatographische Kontrolle des [^{14}C]-Acetateinbaus in die Phosphatidfettsäuren der Sojabohnen. *Z. Anal. Chem.*, 252 (1970) 137–139.

O'CONNOR, R. T. AND HERB, S. F.: Specifications of fatty acid composition for identification of fats and oils by gas–liquid chromatography. *J. Amer. Oil Chem. Soc.*, 47 (1970) 186A, 195A, 197A.

POKORNÝ, J., HLADÍK, J. AND ZEMAN, I.: (The composition of castor oil). *Pharmazie*, 23 (1968) 332–333.

ZHUKOV, A. V. AND VERESHCHAGIN, A. G.: (Heptadecenoic acid as an internal standard in the gas chromatographic determination of the weight of fatty acids). *Zh. Anal. Khim.*, 25 (1970) 2222–2227.

15. TERPENES, ESSENTIAL OILS AND OTHER VOLATILE AROMATIC COMPOUNDS

GLEISPACH, H. AND SCHANDARA, E.: Untersuchungen zur gas chromatographischen Analyse von Menthol aus dem Urin. *Z. Anal. Chem.*, 252 (1970) 140–143.

HARTMANN, N. AND HAWKES, S. J.: Statistical analysis of multiveriate chromatographic data on natural mixtures, with particular reference to peppermint oils. *J. Chromatogr. Sci.*, 8 (1970) 610–611.

MONIR, T. AND TAKÁCZ, J.: (Gas chromatography of Patschouli oil). *Olaj, Szappan, Kozmetika*, 17, No. 2 (1968) 56–58.

16. NITRO AND NITROSO COMPOUNDS

HOFFMANN, D. AND RATHKAMP, G.: Quantitative determination of nitrobenzenes in cigarette smoke. *Anal. Chem.*, 42 (1970) 1643–1647.

RHOADES, J. W. AND JOHNSON, D. E.: Gas chromatography and selective detection of N-nitrosoamines. *J. Chromatogr. Sci.*, 8 (1970) 616–617.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

GOLOVISTIKOV, YU. N.: (Gas chromatographic separation of isomers of toluenediisocyanate). *Zavodsk. Lab.*, 36 (1970) 1313–1314.

18. AMINO ACIDS

KVENVOLDEN, K. A., PETERSON, E. AND BROWN, F. S.: Racemization of amino acids in sediments from Saanich Inlet, British Columbia. *Science*, 169 (1970) 1079–1082.

PARR, W., YANG, C., BAYER, E. AND GILL-AV, E.: Interaction between asymmetric solutes and solvents: N-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester as solvent. *J. Chromatogr. Sci.*, 8 (1970) 591–595.

VANDENHEUVEL, W. J. A., SMITH, J. L. AND COHEN, J. S.: Gas–liquid chromatography and mass spectrometry of carbon-13 enriched and deuterated amino acids as trimethylsilyl derivatives. *J. Chromatogr. Sci.*, 8 (1970) 567–576.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19b. Elucidation of structure of proteins

TSCHESCHE, H., OBERMEIER, R. AND KUPFER, S.: Gaschromatographische Identifizierung von Thiohydantoinen aus dem Abbau von Peptiden und Proteinen. *Angew. Chem.*, 82 (1970) 880–881.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DURBIN, D. E. AND ZLATKIS, A.: A gas chromatographic method for the determination of pyridine and quinoline type bases. *J. Chromatogr. Sci.*, 8 (1970) 608–610.

24. ORGANIC SULPHUR COMPOUNDS

OBOLENTSEV, R. D., MAKOVA, E. A., IBRAGIMOVA, F. SH. AND LYUBOPYTOVA, N. S.: (Group composition of mercaptans from kerosene distillates of petroleum). *Neftekhimiya*, 10 (1970) 899–906.

SCHULZ, H. AND MUNIR, M.: Schwefelverbindungen in Erdöl und Erdölprodukten. *Erdöl Kohle*, 23 (1970) 708–708.

25. ORGANIC PHOSPHORUS COMPOUNDS

BOYDEN, J. W. AND DEACON, M. T.: A gas chromatographic determination of tetraaryl pyrophosphates and diaryl phosphochloridates in triaryl phosphate. *Analyst*, 95 (1970) 935–939.

26. ORGANOMETALLIC AND RELATED COMPOUNDS

JITSU, Y., KUDO, N., SATO, K. AND TESHIMA, T.: (Determination of organotin compounds by gas–liquid chromatography). *Japan Analyst*, 18 (1969) 169–175.

28. ANTIBIOTICS

TSUJI, K. AND ROBERTSON, J. H.: Gas–liquid chromatographic determination of amino-glycoside antibiotics: kanamycin and paromomycin. *Anal. Chem.*, 42 (1970) 1661–1663.

29. INSECTIDES AND OTHER PESTICIDES

ACKER, L. AND SCHULTE, E.: Über das Vorkommen von chlorierten Biphenylen und Hexachlorbenzol neben chlorierten Insektiziden in Humanmilch und menschlichem Fettgewebe. *Naturwiss.*, 57 (1970) 497–497.

KAWAHARA, T., GOTO, S. AND KASHIWA, T.: (Thermionic gas–liquid chromatography of organophosphorus pesticides). *Japan Analyst*, 18 (1969) 698–703.

30. SYNTHETIC AND NATURAL DYES

TERRILL, J. B. AND JACOBS, E. S.: Application of gas–liquid chromatography to the analysis of anthraquinone dyes and intermediates. *J. Chromatogr. Sci.*, 8 (1970) 604–607.

31. PLASTICS AND THEIR INTERMEDIATES

ALEKSEEVA, K. V., KHRAMOVA, L. P. AND STREL' NIKOVA, I. A.: (Application of pyrolysis gas chromatography to analysis of copolymers of vinylaromatic hydrocarbons with aliphatic dienes). *Zavodsk. Lab.*, 36 (1970) 1304–1307.

AST, W. AND HUMMEL, K.: Gaschromatographische Polymer-Analyse nach Abbau durch Metathese. *Naturwiss.*, 57 (1970) 545–545.

KEPKE, V. L. AND SOKOLIN, G. F.: (Control of polymerisation in the production of SK (synthetic rubber) with the aid of an automatic chromatograph). *Zavodsk. Lab.*, 36 (1970) 1301–1304.

TAKEUCHI, T., OKUMOTO, T. AND TSUGE, S.: (Analysis of vinylidene chloride-methylmethacrylate copolymers by means of pyrolysis gas chromatography). *Japan Analyst*, 18 (1969) 614–618.

ZIZIN, V. G., BERDINA, L. KH. AND AVDEEVA, M. P.: Determination of the composition of divinylstyrene copolymers and the polymerisation degree of block copolymers by pyrolysis gas chromatography). *Zavodsk. Lab.*, 36 (1970) 1307–1310.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

BLACKMORE, D. J.: The determination of carbon monoxide in blood and tissue. *Analyst*, 95 (1970) 439–458.

CARLE, G. C.: Gas chromatographic determination of hydrogen, nitrogen, oxygen, methane, krypton, and carbon dioxide at room temperature. *J. Chromatogr. Sci.*, 8 (1970) 550–551.

GNAUCK, G., ROSNER, E. AND EICHHORST, E.: Über die Adsorption von Edelgasen und anderen tiefsiedenden Gasen an synthetischen Zeolithen. *Chem. Tech., (Berlin)*, 22 (1970) 680–685.

JARIWALA, S. L. AND WAKEMAN, I. B.: A gas chromatographic technique for the analysis of hydrocarbon oxidation products. *J. Chromatogr. Sci.*, 8 (1970) 612–614.

VAN DER VLIST, E. AND DE JONG, J. M.: Oxygen–nitrogen separation on Cr_2O_3 gel. *J. Chromatogr.*, 52 (1970) 486–486.

33b. Volatile inorganic substances

BALLSCHMITER, K.: Inorganic trace analysis combining thin-layer and gas chromatography: indirect determination of ruthenium as its thiosemicarbazide chelate. *J. Chromatogr. Sci.*, 8 (1970) 496–498.

BRAZHNIKOV, V. V. AND SAKODYNSKĬ, K. I.: (The gas chromatographic separation of some metal chlorides). *Zh. Prikl. Khim.*, 43 (1970) 2247–2253.

COTTON, T. M., BALLSCHMITTER, K. AND KATZ, J. J.: Gas chromatographic determination of water in chlorophyll. *J. Chromatogr. Sci.*, 8 (1970) 546–549.

DZHIOEVA, Z. K., ZAKHAROV, E. V., ZETKIN, V. I. AND RASKINA, A. D.: Analysis of the products of the synthesis of trichloromethanesulphenyl chloride. *Zh. Anal. Khim.*, 25 (1970) 2252–2253.

SEPPELT, K. AND SUNDERMEYER, W.: Neue Imidoschwefeloxiddifluoride. *Angew. Chem.*, 82 (1970) 931–931.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

SAKODYNSKIĬ, K. I., CHUDYAKOV, V. L. AND YASHIN, YA. I.: (Gas chromatographic separation of isotope-substituted hydrocarbons). *Isotopenpraxis*, 4 (1968) 278–280.

SAKODYNSKIĬ, K. I. VOLKOV, S. A. AND ZELVENSKIĬ, V. YU.: (The separation of labelled compounds by means of preparative gas chromatography). *Isotopenpraxis*, 4 (1968) 305–307.

TING, C. T. AND ROWLAND, F. S.: Recoil tritium reactions with methyl isocyanide and methyl cyanide. Estimates of energy deposition for the T-for-H reaction. *J. Phys. Chem.*, 74 (1970) 4080–4084.

WASIK, S. P. AND TSANG, W.: Determination of trace amounts of contaminants in water by isotope dilution gas chromatography. *Anal. Chem.*, 42 (1970) 1649–1651.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

GALLIARD, T. AND SWOBODA, P. A. T.: Analysis of food constituents by gas chromatography and associated techniques. *Proc. Soc. Anal. Chem.*, 7 (1970) 205–205.

KATNIK, R. J. AND JOHNSON, G. V.: Gas chromatographic analysis of cyclohexane oxidation products. *J. Chromatogr. Sci.*, 8 (1970) 543–545.

LIEBICH, H. M., KOENIG, W. A. AND BAYER, E.: Analysis of the flavor of rum by gas–liquid chromatography and mass spectrometry. *J. Chromatogr. Sci.*, 8 (1970) 527–533.

Electrophoretic Techniques

2. FUNDAMENTALS, THEORY AND GENERAL

CHABARD, J. L., BESSE, G., VOISSIRE, G., PETIT, J. AND BERGER, J. A.: Stability of complexes studied by thin-layer electrophoresis. I. General theory. Application to weak acids. *Bull. Soc. Chim. Fr.*, (1970) 2425–2431; *C.A.*, 73 (1970) 66876d — Paper.

3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

GUSEV, A. I.: Device for the preparative separation of substances by disc electrophoresis in polyacrylamide gel. *Byull. Eksp. Biol. Med.*, 68 (1969) 104–108; *C.A.*, 72 (1970) 75459b — Polyacrylamide gel.

SCHIRMER, H.: (Fixation of paper strips for horizontal electrophoresis). *Ger. Pat.*, 1,298,737 (Cl. G 01 *n*), 03 Jul 1969, Appl. 29 Nov 1962, 4 pp.: *C.A.*, 71 (1969) 74508j.

WINGO, W. J., CARLSON, G. L. AND BORDENCA, C. M.: Apparatus for rapid application of samples in paper chromatography, paper electrophoresis and thin-layer chromatography. *Ala. J. Med. Sci.*, 6 (1969) 464–465; *C.A.*, 72 (1970) 97207v.

WINTERHOFF, D. AND SCHWOEPPE, H.: Simple method for identification of proteins in disc electrophoresis. *Z. Klin. Chem. Klin. Biochem.*, 7 (1969) 309; *Anal. Abstr.*, 19 (1970) 543.

10. CARBOHYDRATES

10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

PANKINA, V. KH.: Electrophoretic separation of seromucoid proteins. *Lab. Delo*, (1970) 183; *C.A.*, 73 (1970) 42285j — starch agar gel.

STEFANOVICH, V. AND AKIYAMA, K.: Comparative studies of aortic acid mucopolysaccharides in fifteen species. *Comp. Biochem. Physiol.*, 34 (1970) 125–130 — acetylated paper.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

BARBIERI, D. AND ALENCASTRO DA SILVA, I.: Urinary urocanic and formiminoglutamic acid determination by a single electrophoretic method and two dye solutions. *Rev. Brasil. Pesqui. Med. Biol.*, 2 (1969) 404–405; *C.A.*, 73 (1970) 63034s — Whatman No. 3 MM paper.

11c. Lipoproteins

FLETCHER, M. J. AND STYLIOU, M. H.: Separating serum lipoproteins by electrophoresis on cellulose acetate. *Clin. Chem.*, 16 (1970) 362–365; *C.A.*, 73 (1970) 64162u.

GAROFF, H., SIMONS, K., EHNHOLM, CH. AND BERG, K.: Demonstration by disc electrophoresis of the lipoprotein carrying the LP(A) antigen in human sera. *Acta Pathol. Micribiol. Scand., Sect. B*, 78 (1970) 253–254; *C.A.*, 73 (1970) 64309x — gel electrophoresis.

NARAYAN, K. A.: Human and rat serum proteins and lipoproteins: ammonium persulphate,gel concentration and disc electrophoresis. *Microchem. J.*, 14 (1969) 335–342; *Anal. Abstr.*, 19 (1970) 1604 — polyacrylamide gel.

SOBOTKA, J. AND TESAROVA-VECHETOVA, A.: Determination of lipoproteins by paper electrophoresis in albumin–buffer solution. *Vnitř. Lék.*, 16 (1970) 397–402; *C.A.*, 73 (1970) 63035t — paper.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

WILSON, C., KIBLER, R. F. AND SHAPIRA, R.: Analysis with trinitrobenzenesulfonic acid of peptides separated on paper. *Anal. Biochem.*, 35 (1970) 371–376; *C.A.*, 73 (1970) 63027s — paper.

19b. Elucidation of structure of proteins

MARDER, V. J., JAMES, H. L. AND SHERRY, S.: Purification of fibrinogen degradation products by Pevikon block electrophoresis. *Thromb. Diath. Haemorrh.*, 22 (1969) 234–239; *C.A.*, 72 (1970) 107684q — Pevikon.

SCHOENMAKERS, J. G. G., GERDING, J. J. T. AND BLOEMENDAL, H.: The subunit structure of α-crystallin isolation and characterization of the S-carboxymethylated acidic subunits from adult and embryonic origin. *Eur. J. Biochem.*, 11 (1969) 472–481 — polyacrylamide gel.

20. PROTEINS (INCLUDING ENZYMES)

PIHAR, O.: (Isoelectrical focusing of proteins). *Chem. Listy*, 64 (1970) 274–281; *C.A.*, 72 (1970) 101061n.

WILKINS, D. J. AND MYERS, P. A.: Anomalous electrophoretic behaviour of certain adsorbed protein derivatives. *Proc. Soc. Exp. Biol. Med.*, 133 (1970) 255–259; *C.A.*, 72 (1970) 97219a.

20a. Proteins of plant origin including bacteria

ALEXANDRESCU, V. AND CALIN, I.: (Electrophoretic and immunoelectrophoretic study of corn isoperoxidases). *Roum. Biochim.*, 6 (1969) 171–178; *C.A.*, 72 (1970) 97360q — agar gel.

BETAIL, G., COULET, M. AND GUILLOT, J.: (Effect of urea on the hemagglutinin of *Pisum sativum*). *C.R. Soc. Biol.*, 163 (1970) 1771–1775; *C.A.*, 73 (1970) 63167n.

ESPOSITO, V. M. AND FEELEY, J. C.: Effect of formalin treatment on electrophoretic mobility of cholera toxin. *Infect. Immunity*, 2 (1970) 120–122; *C.A.*, 73 (1970) 62625y — polyacrylamide gel.

HUEBNER, F. R.: Comparative studies on glutenins from different classes of wheat. *J. Agr. Food Chem.*, 18 (1970) 256–259; *C.A.*, 72 (1970) 96733b.

KOSOUROV, G. I., POGLAZOV, B. F. AND NIKOL'SKAYA, T. I.: (Molecular organization of the tail core of T2 bacteriophage). *Biokhimiya*, 35 (1970) 419–421; *C.A.*, 73 (1970) 12510d.

NEWKIRK, J. D. AND HULCHER, F. H.: Isolation and properties of a fluorescent pigment from *Pseudomonas mildenbergii*. *Arch. Biochem. Biophys.*, 134 (1969) 395–400 — polyacrylamide gel.

20b. Plasma proteins

BRUMBY, P. E. AND WELCH, V. A.: Fractionation of bovine serum lipoproteins and their characterization by gradient gel electrophoresis. *J. Dairy Res.*, 37 (1970) 121–128; *C.A.*, 72 (1970) 96765p — paper and polyacrylamide gel.

DECLEIR, W. AND RICHARD, A.: A study of the blood proteins in *Sepia officinalis* L. with special reference to embryonic hemocyanin. *Comp. Biochem. Physiol.*, 34 (1970) 203–211 — agar gel.

HAYES, D. K., MENSING, E. AND SCHECHTER, M. S.: Electrophoretic patterns of proteins in hemolymph obtained from the adult Madeira cockroach, *Leucophaea maderae* (F), during a twenty-four hour period. *Comp. Biochem. Physiol.*, 34 (1970) 733–737 — polyacrylamide gel.

KARLSSON, B. W.: Fetoprotein and albumin levels in the blood serum of developing neonatal pigs. *Comp. Biochem. Physiol.*, 34 (1970) 535–546 — agar gel, immunoelectrophoresis.

KLEINT, V. AND WEGNER, H.: (Electrophoretic studies on human plasma cryoprecipitates with high antihaemophilic globulin A contents). *Z. Ges. Inn. Med. Ihre Grenzgeb.*, 25 (1970) 312–314; *C.A.*, 73 (1970) 64353g — paper.

MAREK, M.: Effect of actinomycin D on synthesis of "cooling protein" in haemolymph of pupae of *Galleria mellonella* L. *Comp. Biochem. Physiol.*, 34 (1970) 221–227 — starch gel.

PESCH, G.: Plasma proteins variation in a winter flounder (*Pseudopleuronectes americanus*) population. *J. Fish. Res. Board Can.*, 27 (1970) 951–954; *C.A.*, 73 (1970) 42636z — cellulose acetate.

SHAUGHNESSY, P. D.: Serum proteins of two sibling species of giant petrel (*macronectes* ssp.). *Comp. Biochem. Physiol.*, 33 (1970) 721–723 — starch gel.

20c. Structural proteins

FRATER, R.: Artifacts in isoelectric focusing. *J. Chromatogr.*, 50 (1970) 469–474 — polyacrylamide gel isoelectric focusing.

20d. Protamines, histones and other nuclear proteins

KONGSVIK, J. R. AND MESSINEO, L.: The isolation and characterization of deoxyribonucleoproteins from human spleen. *Arch. Biochem. Biophys.*, 136 (1970) 160–166 — cellulose acetate.

MOHBERG, J. AND RUSCH, H. P.: Isolation of the nuclear histones from the myxomycete, *Physarum polycephalum*. *Arch. Biochem. Biophys.*, 134 (1969) 577–589 — polyacrylamide gel.

20e. Chromoproteins

BROWN, I. R. F., BANNISTER, W. H. AND DE LUCCA, C.: A comparison of Maltese and Silician sparrow haemoglobins. *Comp. Biochem. Physiol.*, 34 (1970) 557–562 — cellulose acetate.

FISCHL, J., EICHHORN, F., RUTTENBERG, A. AND MAJOR, CH.: Convention and preparative electrophoretic separation of some urinary porphyrins and porphyrin precursors. *Clin. Chem.*, 16 (1970) 331–334; *C.A.*, 73 (1970) 63029u — membrane filter.

GEORGE, D. W. AND DESSAUER, H. C.: Immunological correspondence of transferrins and the relationships of colubrid snakes. *Comp. Biochem. Physiol.*, 33 (1970) 617–627 — immunoelectrophoresis.

GUTTMAN, S. I.: Hemoglobin electrophoresis and relationships within the lizard genus *Sceloporus* (Saurina: Iguanidae). *Comp. Biochem. Physiol.*, 34 (1970) 563–568 — starch gel.

GUTTMAN, S. I.: An electrophoretic study of the hemoglobins of the sand lizards, *Callisaurus*, *Cophosaurus*, *Holbrookia* and *Uma*. *Comp. Biochem. Physiol.*, 34 (1970) 569–574 — starch gel.

JOHANNSEN, B., GES, J. AND KNOLL, P.: Electrochemical marking of ferritin by iodine-131. *Wiss. Z. Karl-Marx Univ. Leipzig, Math.-Naturwiss. Reihe*, 18 (1969) 593–594; *C.A.*, 72 (1970) 97237e — polyacrylamide gel, paper.

MÄSIAR, P.: On hemoglobin. XXVI. Hemoglobin of hedgehog (*Erinaceus roumanicus*). I. *Comp. Biochem. Physiol.*, 33 (1970) 697–703 — starch gel.

POLUHOWICH, J. J.: An electrophoretic comparison of hemoglobins from American and European eels. *Comp. Biochem. Physiol.*, 34 (1970) 739–743 — starch gel.

RAPI, G., FRANGINI, V., MOGGI, C., PRATESI, C. AND TESTAFERRATA, A.: Radial electrophoresis on cellulose acetate gel for the study of human hemoglobin. *Riv. Clin. Pediat.*, 81 (1968) 1199–1204; *C.A.*, 73 (1970) 42286k — cellulose acetate.

SORCINI, M., ORLANDO, M. AND TENTORI, L.: Polymorphism of hemoglobin of *Triturus cristatus*. *Comp. Biochem. Physiol.*, 34 (1970) 751–753 — polyacrylamide gel.

WIYGUL, G., MITLIN, N., LOVE, J. N. AND LUSK, G. J.: The absorption and metabolism of [U-^{14}C]-glycine in the irradiated and normal boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae). *Comp. Biochem. Physiol.*, 33 (1970) 475–480 — cellulose acetate.

ZAGALSKY, P. F., CECCALDI, M. J. AND DAUMAS, R.: Comparative studies on some decapod crustacean carotenoproteins. *Comp. Biochem. Physiol.*, 34 (1970) 579–607 — Starch gel.

20f. Varia, with special reference to non-identified and tissue proteins

AZERAD, E., LEWIN, J., LUBETZKI, J., DUPREY, J., MILLION, M. AND FRIEDLER, D.: Evidence of the heterogeneity of crystalline insulin by electrophoresis in polyacrylamide gel. *Diabetes*, 15 (1967) 275–280; *C.A.*, 73 (1970) 62858b — polyacrylamide gel.

CALHOUN, W. B. AND KOENIG, L.: The distribution of the soluble proteins in the lenses of some marine vertebrates. *Comp. Biochem. Physiol.*, 34 (1970) 71–80 — cellulose acetate, free flow electrophoresis.

DALES, R. P. AND PELL, J. S.: The nature of the peritrophic membrane in the gut of the terebellid polychaete *Neoamphitrite figulus*. *Comp. Biochem. Physiol.*, 34 (1970) 819–826 — cellulose acetate.

DOMIC-PEREDO, N.: (Immunoelectrophoretic analysis of cerebrospinal fluid protein). *Farm. Glas.*, 26 (1970) 163–174; *C.A.*, 73 (1970) 64238y — paper.

MORENKOVA, S. A. AND BABICH, P. A.: (Complexing of the A-chain of insulin with glycine). *Dokl. Akad. Nauk SSSR*, 191 (1970) 1417–1419; *C.A.*, 73 (1970) 62623w — polyacrylamide gel.

OKAMOTO, R., ASHIKAGA, K. AND OHTSUKA, H.: Isolation of prolactin by acrylamide gel electro phoresis of the rat pituitary tissue culture medium. *Bull. Tokyo Med. Dent. Univ.*, 16 (1969) 343–349; *C.A.*, 73 (1970) 42100v — polyacrylamide gel.

SMITH, A. C.: Permeability of the eye lens capsule of the bigeye tuna to nuclear eye lens proteins. *Comp. Biochem. Physiol.*, 34 (1970) 101–108 — paper.

WINDISCH, R. M. AND BRACKEN, M. M.: Cerebrospinal fluid proteins; concentration by membrane ultrafiltration and fractionation by electrophoresis on cellulose acetate. *Clin. Chem.*, 16 (1970) 416–419; *C.A.*, 73 (1970) 63030n — cellulose acetate.

20g. Enzymes: oxidoreductases

COLES, G. C.: A comparison of some isoenzymes of *Schitosoma mansoni* and *Schitosoma haematobium*. *Comp. Biochem. Physiol.*, 33 (1970) 549–558 — polyacrylamide gel.

MCCABE, M. M., DEAN, D. M. AND OLSON, G. S.: Multiple forms of 6-phosphogluconate dehydrogenase in the skipjack tuna, *Katsuwonus pelamis*. *Comp. Biochem. Physiol.*, 34 (1970) 755–757 — starch gel.

MORALES HALVA, J. A., VALEGA HAGASICH, A., URIBE ECHEVARRIA, J. M., SAPAG HAGAR, M. and VARLETA CORTEZ, R.: (Role of serum proteins in the electrophoretic migration of isoenzymes). *Langre*, 14 (1969) 427–441; *C.A.*, 73 (1970) 41984z — paper.

MURPHY, M. J.: Starch-iodide as a strain for peroxidases. *J. Chromatogr.*, 50 (1970) 539–540 — starch gel.

20h. Enzymes: transferases

TSOLAS, O. AND HORECKER, B. L.: Isoenzymes of transaldolase in *Candida utilis*. I. Isolation of three isoenzymes from yeast extracts. *Arch. Biochem. Biophys.*, 136 (1970) 287–302 — polyacrylamide gel.

20i. Enzymes: hydrolases

ALEXANDRESCU, V. AND MIHAILESCU, F.: Electrophoretic study of wheat amylases during the first growing stages. *Rev. Roum. Biochim.*, 7 (1970) 63220z — agar gel.

ANJIC, J., HAYEM, A., CARLIER, A. AND BONTE, M.: Isolation of salivary amylase isoenzymes by isoelectric focusing. *C. R. Acad. Sci. (Paris), Ser. D*, 270 (1970) 407–409; *C.A.*, 72 (1970) 96857v.

BECKENDORF, G. W. AND STEPHEN, W. P.: The effect of aging on the multiple molecular esterase forms taken from tissues of *Periplaneta americana* (L.). *Biochim. Biophys. Acta*, 201 (1970) 101–108 — polyacrylamide gel.

DANDRIFOSSE, G.: Mechanism of amylase secretion by the pancreas of the pigeon. *Comp. Biochem. Physiol.*, 34 (1970) 229–235 — polyacrylamide gel.

DELINCÉE, H. AND RADOLA, B. J.: Some size and charge properties of tomato pectin methylesterase. *Biochim. Biophys. Acta*, 214 (1970) 178–189 — thin-layer electrofocusing, paper.

EPSTEIN, E., WOLF, P. L. AND ZAK, B.: Study of the indigogenic detection of serum acid phosphatase in polyacrylamide gel electrophoresis. *Enzymologia*, 35 (1968) 257–262; *Anal. Abstr.*, 18 (1970) 1928 — polyacrylamide gel.

GREGORATTI, CH. L. AND MILLS, R. R.: Midgut epithelium of the American cockroach: induced synthesis of acid phosphomonoesterase by starvation. *Comp. Biochem. Physiol.*, 34 (1970) 345–353 — polyacrylamide gel.

KINGSBURY, N. AND MASTERS, C. J.: Molecular weight interrelationships in the vertebrate esterases. *Biochim. Biophys. Acta*, 200 (1970) 58–69 — polyacrylamide gel.

MCCABE, M. M. AND DEAN, D. M.: Esterase polymorphisms in the skipjack tuna, *Katsuwonus pelamis*. *Comp. Biochem. Physiol.*, 34 (1970) 671–681 — starch gel.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

GANGLOFF, J., KEITH, G. AND DIRHEIMER, G.: Separation and analysis of an oligonucleotide mixture. *Bull. Soc. Chim. Biol.*, 52 (1970) 125–133; *C.A.*, 73 (1970) 63028t.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

JOHRI, B. N.: Thin-layer electrophoresis of indole derivatives. *J. Chromatogr.*, 50 (1970) 340–344 — Silica gel G plates.

28. ANTIBIOTICS

GARBER, C. AND DOBRECKY, J.: (Paper electrophoresis of antibiotics. I. Derivatives of amino sugars and polypeptides). *Rev. Asoc. Bioquim. Argent.*, 33 (1968) 180–184; *Anal. Abstr.*, 18 (1970) 2728.

33. INORGANIC SUBSTANCES

DREVER, J. I.: Electrophoresis and the study of clay minerals by continuous particle electrophoresis. Geochemical study of the clay fractions of the sediments of the Rio America, Mexico. *Diss. Abstr., Int. B*, 30 (1969) 254; *C.A.*, 72 (1970) 92303p.

JOKL, V. AND PIKULIKOVÁ, Z.: Densitometrische Bestimmung von Fe(II)–Fe(III)-Gemischen nach elektrophoretischer Trennung mittels N-(2-Hydroxyäthyl)iminodiessig-säure. *J. Chromatogr.*, 50 (1970) 540–543 — paper.

LEDERER, M., MORPURGO, L. AND OSSICINI, L.: A paper electrophoretic study of ion pair formation. V. Paper electrophoresis and anion-exchange paper chromatography of inorganic anions with various electrolytes. *J. Chromatogr.*, 50 (1970) 475–481 — Whatman No. 1 paper.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

OYOSHI, E., OYOSHI, A. AND SHINAGAWA, M.: Radiochemical determination of several nuclides for uranium-235 fission. *Radiochem. Radioanal. Lett.*, 3 (1970) 1–5; *C.A.*, 72 (1970) 95905x — paper.

RONQUIST, G. AND AGREN, G.: Isolation of ^{32}P-labeled phosphorylthreonine from Ehrlich mous ascites tumor cells suspended in an isotonic medium containing ^{32}P-labeled adenosine triphosphate. *Acta Chim. Scand.*, 24 (1970) 728–729; *C.A.*, 73 (1970) 41800m — paper.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

LAJOLO DI COSSANO, D., ASTALDI, Jr., A., PECCO, P., BERT, G. AND ASTALDI, G.: Electrophoretic mobility of human lymphocytes in the presence of *Escherichia coli* L-asparaginase. *Exp. Cell Res.*, 60 (1970) 458–459.

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Errata

J. Chromatogr., 57 (1971) 173–180

Figures occurring on p. 174 and on p. 176 should be interchanged.

J. Chromatogr., 58 (1971) 19–30

Page 23. Table II, headings to columns 3, 5, 7 and 9 should read: $V_g/V_{g(C_5H_{12})}$.

J. Chromatogr., 59 (1971) 269–279

Page 276, 8th line up and page 277, 8th line below Table VI, "eqn. 7" should read "eqn. 8".