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REDISTRIBUTION REACTIONS INVOLVING TWO DIFFERING CENTRAL ATOMS

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SUMMARY

This paper considers the exchange of alkyl and ester groups between two different central atoms. Confirmation of the exchange of methoxy and ethoxy groups on silicon and phosphorus alone was first established. Then follows a description of attempts to induce:

(i) Exchange of these ester groups between silicon and phosphorus;

(ii) exchange of methyl and ethoxy groups between tin and silicon, and between tin and phosphorus;

(iii) exchange of alkyl and ethoxy groups on silicon.

Only a limited amount of exchange was observed in most of the systems examined.

INTRODUCTION

Redistribution, rearrangement, exchange or scrambling reactions were first recognised by CALINGAERT[1-7] who examined alkyl interchange in organo-silicon, tin, lead and mercury compounds. A great many examples of such reactions have been observed, especially in groups II–VII among the non-transitional elements. Thermodynamic, kinetic and mechanistic characteristics have received detailed attention in certain systems. Investigations up to 1963 have been reviewed by LOCKHART[8], and more recently by MOEDRITZER[9], whilst other papers of interest have also appeared[10,11].

Several types of reaction are possible:

(A) Intermolecular

(i) Where the central atom is the same and the substituents are different.

$$MX_n + MY_n \rightleftharpoons MX_{n-1}Y + \ldots . + MXY_{n-1}$$

(ii) Where the central atoms are different, but the substituents are the same.

$$MX_n + NX'_m \rightleftharpoons MX_{n-1}X' + \ldots . + NX'_{m-1}X$$

(iii) Where the central atoms and substituents are different.

$$MX_n + NY_m \rightleftharpoons MX_{n-1}Y + \ldots . + NY_{m-1}X + MY_n + NX_m.$$

(B) Intramolecular

Exchange between two stereochemically distinct positions in a molecule.

Types A(ii) and A(iii) are used in preparative chemistry[12,13]. However, type A(i) comprises the largest number of systems so far studied. The work described in this paper is concerned with rearrangements of the A(iii) type.

The use of chromatography for analysis of redistribution reactions has been somewhat limited, the work of RUSSELL[14–16] being a notable exception. This neglect is difficult to understand, since chromatography usually provides a rapid and accurate means of quantitative and qualitative analysis of reaction mixtures. The Griffin and George D6 chromatograph, with gas-density balance detector was used throughout the work described in this publication.

Redistributions between two different central atoms have not been studied to any great extent, and very few examples are to be found in the literature[17–20].

There seems no obvious reason why redistribution should not occur between two different atoms provided that it occurs on each atom individually. POLLARD, NICKLESS AND UDEN[10] observed that in the same group (IV B), alkyl exchange occurs only where central atoms are adjacent members of the group.

Redistributions of ester groups on silicon[21] and phosphorus[22] have been studied. This suggested a starting part for the results presented in this paper; *i.e.* is it possible to induce exchange of ester groups between these two central atoms?

EXPERIMENTAL

Methyl phosphate, ethyl phosphate and ethyl silicate used were the commercially available materials. However, in order to complete the experiments, methyl silicate was required and this material is not readily available. Thus it was necessary to prepare the pure ester and unfortunately this proved to be more difficult than expected, and the synthesis is given in some detail.

Preparation of methyl silicate

The methods described by EMBLEM[23], and PEPPARD *et al.*[24], were tried, but neither proved at all satisfactory, since in both preparations the product was contaminated with HCl, the last traces of which were very difficult to remove. The problem was eventually solved by using a modification of the PEPPARD method:

$$4CH_3OH + SiCl_4 \rightarrow Si(OMe)_4 + HCl$$
(20% excess)

The reaction was carried out on one-quarter molar scale; 45 ml of calcium-dried methanol were cooled in solid CO_2 in a 250 ml flask fitted with a silica gel drying tube, and containing a small piece of calcium. Redistilled $SiCl_4$ (29 ml) was added as quickly as possible with vigorous shaking, followed by a few ml of a high-boiling petrol oil.

The mixture was then refluxed for 2 h while pure, dry nitrogen was bubbled through the system. In this way most of the HCl was removed from the reaction. Pure triethylamine was then added till the solution was slightly alkaline (*ca.* 1 ml was required) and the white triethylamine hydrochloride filtered off. The filtrate was fractionally distilled using a 2 ft. column packed with glass helices. Pure methyl silicate was obtained in 75% yield from the first fractionation, b.p. 120°. It was stored in dry glass apparatus, stoppers being fitted with PTFE sleeves.

Redistributions involving esters of silicon and phosphorus

System A: $O{=}P(OMe)_3 + O{=}P(OEt)_3$

This system has been examined in some detail by MOEDRITZER *et al.*[22], who observed that for mixtures of trialkyl phosphates with or without HCl as catalyst, no redistribution was observed after 3 months at room temperature. With trace amounts of $NaOCH_3$, equilibrium was achieved after 6 days at 120° in sealed tubes. Under the same conditions with no catalyst, there was no sign of rearrangement. Redistribution was observed after 7 days at 200° in the absence of catalyst, however, in a sealed system. The formation of volatile products (*e.g.* dimethyl ether) was observed under these conditions. Equilibrium data, calculated on the basis of quantitative N.M.R. measurements are also presented[22].

System A was examined by the present authors under two distinct sets of conditions, an equimolar mixture of reactants was used throughout.

Experiment A1. Reactants were heated together at atmospheric pressure for 18 h at 120° in the presence of a catalytic amount of pure $AlCl_3$. In no case was any rearrangement observed.

Experiment A2. Reactants were sealed in a glass tube with a trace of $NaOCH_3$, and heated for 7 days at 120°. GLC analysis showed that redistribution was complete (Fig. 1 and Table I).

Column temperature: 150°; column: 25% silicone oil/celite 60–85;
p_i/p_o (N_2), 1.6.

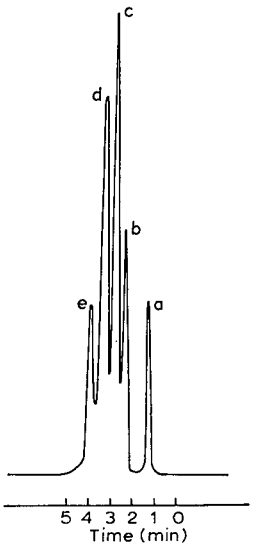
TABLE I

RESULTS OF EXPERIMENT A2

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$O{=}P(OMe)_3$	1.93
c	$O{=}P(OMe)_2(OEt)$	2.40
d	$O{=}P(OMe)(OEt)_2$	2.91
e	$O{=}P(OEt)_3$	3.48

System B: $Si(OMe)_4 + Si(OEt)_4$

This system has been examined by MOEDRITZER AND VAN WAZER[21], who claim that equilibrium is established after 10 days at 150°, in sealed tubes in the absence of catalyst. A trace of the GLC analysis of the final reaction mixture has been published[25].



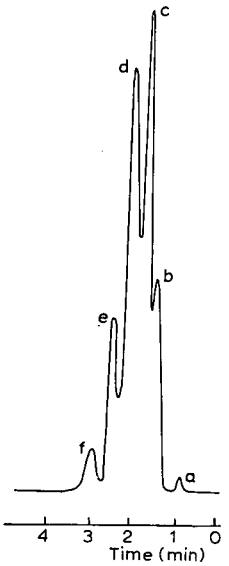


Fig. 1. Experiment A2. Reaction between O = $P(OMe)_3$ and O = $P(OEt)_3$. (a) = Diethyl ether, (b) = O = $P(OMe)_3$, (c) = O = $P(OMe)_2(OEt)$, (d) = O = P(OMe) $(OEt)_2$, (e) = O = $P(OEt)_3$.

Fig. 2. Experiment B1. Reaction between $Si(OMe)_4$ and $Si(OEt)_4$. (a) = Diethyl ether, (b) = $Si(OMe)_4$, (c) = $Si(OMe)_3(OEt)$, (d) = $Si(OMe)_2(OEt)_2$, (e) = Si(OMe) $(OEt)_3$, (f) = $Si(OEt)_4$.

Experiment B1. Reactants were sealed in glass tubes in the absence of a catalyst and placed in an oven at 120°. Tubes were opened and the reaction mixture examined by GLC at daily intervals, instrument data as for System A.

It was found that under these conditions, equilibrium occurs in less than 2 days, and consideration of the relative peak sizes indicates that redistribution is ideal (*i.e.* perfectly random). The trace of the equilibrium mixture is shown in Fig. 2 (see also Table II).

TABLE II

RESULTS OF EXPERIMENT B1

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$Si(OMe)_4$	1.21
c	$Si(OMe)_3(OEt)$	1.60
d	$Si(OMe)_2(OEt)_2$	1.76
e	Si(OMe) $(OEt)_3$	2.11
f	$Si(OEt)_4$	2.63

Comparison of Fig. 2 with the results obtained by MOEDRITZER *et al.*[22], indicates that in the latter case equilibrium has clearly not been attained.

System C: $O{=}P(OEt)_3 + Si(OMe)_4$

If rearrangement in this system occurs ideally, it should give rise to nine possible products, *i.e.* five mixed esters of silicon, plus four mixed esters of phosphorus. The reaction was investigated using five distinct sets of experimental conditions. Where applicable the reaction mixtures were examined by GLC (instrument data as in system A).

Experiment C1. Reactants were mixed and sealed in glass tubes with a trace of $NaOCH_3$. The tubes were heated in an oven at 120° for seven days. After this time the contents of each tube appeared to be completely gaseous, and so before opening, the tubes were immersed in liquid nitrogen for a few sec. On warming to room temperature, the contents of each tube evaporated rapidly, leaving a small amount of solid residue, which was extracted with ether and examined by GLC. In no case were peaks corresponding to either starting material observed.

It is apparent that under these conditions the esters decompose to give volatile hydrocarbons or ethers, and consequently, less rigorous conditions were used for the succeeding experiments.

Experiment C2. Reactants were sealed in glass tubes in the absence of a catalyst, and heated in an oven at 120°. Tubes were opened at intervals up to 4 weeks, at which time the contents of all remaining tubes were solid. Reaction mixtures were examined by GLC (data as in system A) for those tubes in which there remained some liquid at room temperature after the volatile components had been allowed to evaporate.

It was found that up to *ca.* 14 days, slight redistribution does occur, and that from that time further heating causes decomposition of the reaction mixture. A trace of the analysis of the reaction mixture after 14 days is shown in Fig. 3 (see also Table III).

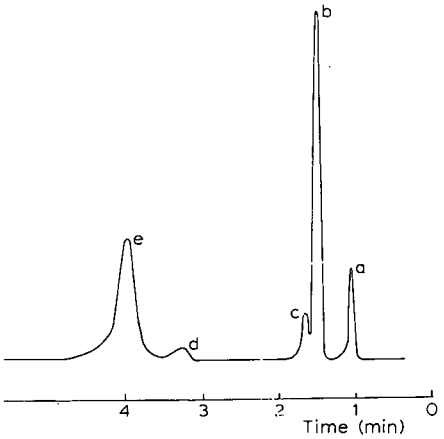


Fig. 3. Experiment C2. Reaction between $O{=}P(OEt)_3$ and $Si(OMe)_4$. (a) = Diethyl ether, (b) = $Si(OMe)_4$, (c) = $Si(OMe)_3(OEt)$, (d) = $O{=}P(OEt)_2(OMe)$, (e) = $O{=}P(OEt)_3$.

Experiment C3. Reactants were sealed in glass tubes in the absence of a catalyst, and placed in an oven at 180°. Tubes were opened at intervals up to 14 days, at which time the contents of all remaining tubes were solid.

In no case was any evidence of rearrangement observed, the decomposition of the starting materials apparently occurring preferentially.

TABLE III

RESULTS OF EXPERIMENT C2

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$Si(OMe)_4$	1.21
c	$Si(OMe)_3(OEt)$	1.63
d	$O=P(OEt)_2(OMe)$	2.91
e	$O=P(OEt)_3$	3.48

Experiment C4. Reactants were heated together at atmospheric pressure in UDEN's redistribution apparatus[10] at 120° in the presence of a trace of $SiCl_4$. Samples were taken at daily intervals up to 20 days, after which time the reaction mixture was solid. GLC analysis revealed no trace of any rearrangement in any of the samples examined.

Experiment C5. Reactants were sealed in glass tubes with a trace of $SiCl_4$, and heated in an oven at 120°. Tubes were opened at intervals up to 14 days and the contents examined by GLC. In no case was any redistribution observed, but a considerable amount of Et_2O was produced.

System D: $O=P(OMe)_3 + Si(OEt)_4$

This system proved to be very difficult to examine by GLC, since on all the stationary phases tried, the separation factor was very small. Although it was possible to increase it to about 1.2 by suitable arrangement of operating parameters the identification of intermediate peaks was rendered impossible. However, the reaction was investigated under similar experimental conditions to system C, to determine whether any parallel existed between the two systems.

Experiment D1. Experimental conditions were as for Experiment C1, *i.e.* reactants sealed with a trace of $NaOCH_3$ at 120° for 7 days, and in general the same effects were observed, *e.g.* on opening the tubes the contents evaporated rapidly, leaving a solid residue which did not contain any of the starting materials.

Experiment D2. Conditions were as for Experiment C2. In this case system D had a propensity for exploding, often with considerable violence, within a few hours of being placed in the oven. In the few cases where tubes were recovered intact, no rearrangement was observed up to 7 days, this being the longest time that a tube of Experiment D2 survived without explosion under these conditions.

Experiment D3. After the spectacular but uninformative results of Experiment D2, rather milder conditions were used for the final experiment in system D.

The reactants were heated together at atmospheric pressure in the absence of a catalyst (and later with a trace of $SiCl_4$) at 120° for up to 10 days. Samples taken at regular intervals showed on GLC analysis that a small amount of Et_2O was formed under these conditions, but redistribution was not observed.

Redistributions involving other central atoms

After the relative failure of attempts to induce ester interchange between silicon and phosphorus, it was decided to replace one of the esters with tetramethyltin, since

tin tetraalkyls undergo rearrangement much more readily than do silicon esters and alkyls.

System E: $SnMe_4 + O{=}P(OEt)_3$

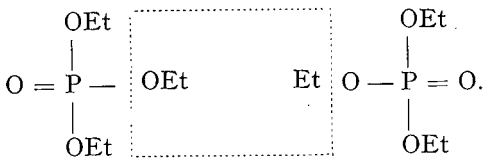
Experiment E1. Reactants were sealed in glass tubes in the absence of a catalyst and heated in an oven at 120°. Tubes were opened at weekly intervals up to 8 weeks, and the contents examined by GLC. In no case was any rearrangement observed.

Experiment E2. Reactants were sealed in glass tubes in the absence of a catalyst and heated at 150°. Tubes were opened at weekly intervals up to 8 weeks and the contents examined by GLC. Although no redistribution was observed, two interesting points were noted:

(i) Each sample showed a small peak corresponding to Et_2O, which became larger as the reaction time increased.

(ii) After about 2 weeks the reaction mixture became viscous, the viscosity increasing with time.

It is suggested that the viscosity is due to the condensation of ethyl phosphate with elimination of ether, thus building up phosphorus–oxygen–phosphorus chains:



Experiment E3. Reactants were sealed in glass tubes in the absence of a catalyst and heated at 180° for 14 days. Although several tubes exploded under these conditions, GLC examination of those surviving revealed that extensive reaction had occurred. A trace of the analysis is shown in Fig. 4.

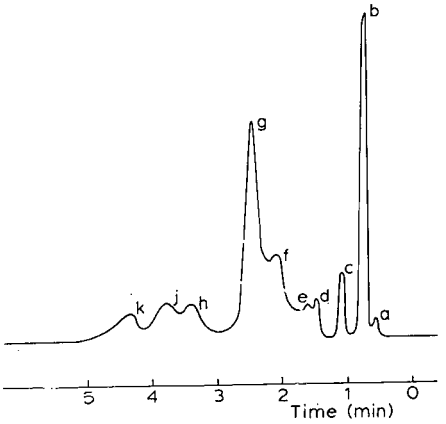


Fig. 4. Experiment E3. Reaction between $O{=}P(OEt)_3$ and $SnMe_4$. (a) = Diethyl ether, (b) = $SnMe_4$, (k) = $O{=}P(OEt)_3$, (c) to (j) = unknowns, (j) could be $O{=}P(OEt)_2(Me)$.

Column temperature: 150°; column: 20% silicone oil/celite 60–85; $p_i/p_o(N_2)$ = 1.6.

Assuming that redistribution of the phosphorus ester occurs only by fission of P–O bonds, and not by fission of O-alkyl bonds, there should be nine possible products of rearrangement, corresponding to a redistribution of methyl and ethoxy groups on both tin and phosphorus. Inspection of Fig. 4 reveals a total of ten peaks, and by comparison with the retention data of known compounds, some are identifiable. (See Table IV.)

TABLE IV

RESULTS OF EXPERIMENT E3

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$SnMe_4$	1.30 (5)
c	Unknown	1.80
d	Unknown	2.65
e	Unknown	2.66
f	Unknown	3.34
g	Unknown	3.87
h	Unknown	5.28
j	Unknown	6.05
k	$O=P(OEt)_3$	7.02

The remaining seven peaks probably correspond to mixed methyl–ethoxy compounds of tin or phosphorus. However, it is possible to state that none of the peaks correspond to a mixed methyl–ethyl tin compound, since under the same GLC conditions the retention ratio (w.r.t. Et_2O) for this series are:

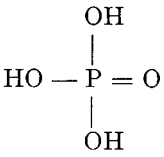
$SnMe_4$	1.30 (5)
$SnMe_3Et$	2.00
$SnMe_2Et_2$	2.90
$SnMeEt_3$	4.26
$SnEt_4$	6.60

Thus, it appears that rearrangement of the phosphorus ester does occur by fission of the P–O bond.

In the E3 experiments, a glassy solid was formed in the reaction tubes, which was extracted with distilled water and examined:

(i) The solution gave a positive reaction for ionic orthophosphate.

(ii) Examination by thin-layer chromatography indicated the presence of monophosphate ions (PO_4^{3-}), and since the solution was acidic, the phosphate is probably in the form:



Experiment E4. Reactants were sealed in glass tubes with a trace of $AlCl_3$ and heated at 180°. In all cases the reactants became solid within a few hours. An ether

extract showed the absence of any starting materials or rearranged products on GLC analysis.

Experiment E5. Reactants were sealed in glass tubes with a trace of $AlCl_3$ and heated at 120°. The results were the same as for Experiment E4.

System F: $SnMe_4$ + $Si(OEt)_4$

Experiment F1. It was quickly discovered that reactants in system F had a considerable tendency to explode when heated in sealed tubes above 120°. Consequently the first experiment in this system consisted of prolonged heating at the maximum temperature (120°) at which explosion did not occur (in the absence of a catalyst).

Tubes were opened at weekly intervals up to ten weeks and the contents examined. In no case was any redistribution observed, nor was any ether formed under these conditions.

Experiment F2. Reactants were heated at atmospheric pressure in the absence of a catalyst at 120°. No rearrangement was observed after 30 days.

Experiment F3. Reactants were heated at atmospheric pressure with a trace of $AlCl_3$ at 120°. Samples were taken at intervals up to 4 days and examined by GLC (instrument data as for E3).

A selection of traces from Experiment F3 is shown in Fig. 5 (See also Table V.)

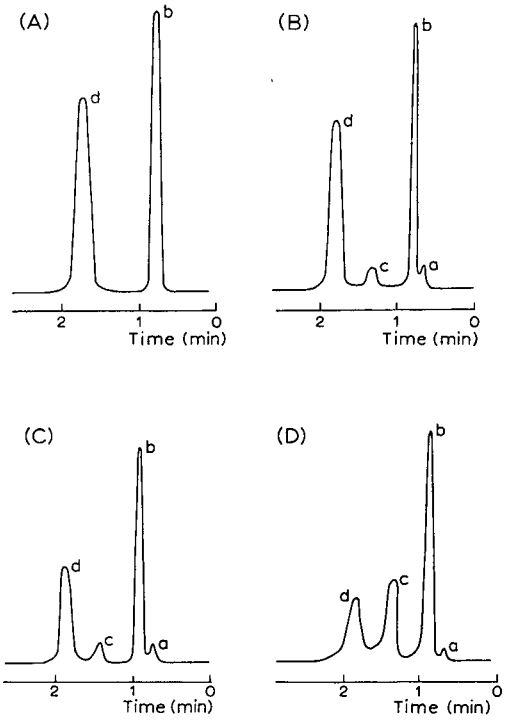


Fig. 5. Experiment F3. (A) at 20 min; (B) at 1 h; (C) at 4 h; (D) at 70 h. Reaction between $Si(OEt)_4$ and $SnMe_4$. (a) = Diethyl ether, (b) = $SnMe_4$, (c) = unknown (probably $Si(OEt)_2Me_2$), (d) = $Si(OEt)_4$.

TABLE V

RESULTS OF EXPERIMENT F3

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$SnMe_4$	1.30 (5)
c	Unknown	2.16
d	$Si(OEt)_4$	3.08

It is noticeable that peak d decreases in size with time; in fact after 10 days the $Si(OEt)_4$ disappeared entirely from the reaction mixture. This is in accordance with the formation of Et_2O during the reaction.

Experiment F4. Reactants were heated at atmospheric pressure with a trace of $SiCl_4$ at 120°. Samples were taken at intervals for GLC analysis (data as for E3). Two specimen traces are shown in Fig. 6. (See also Table VI.)

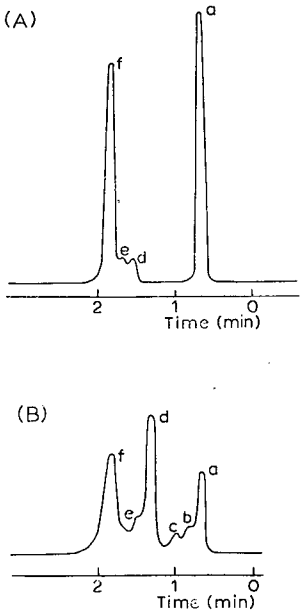


Fig. 6. Experiment F4. (A) at 20 h; (B) at 6 days. Reaction between $Si(OEt)_4$ and $SnMe_4$. (a) = $SnMe_4$, (b), (c), (d), and (e) are unknowns, (f) = $Si(OEt)_4$; (d) is probably $Si(OEt)_2Me_2$, (e) is probably $Sn(OEt)_2Me_2$, (b) and (c) are possibly $Si(OEt)Me_3$ and $Sn(OEt)Me_3$.

As in Experiment F3, the $Si(OEt)_4$ gradually disappeared from the reaction mixture, none being detected after 14 days. It is interesting to note that peak d in F4 corresponds to c in F3.

Since tin alkyls do not rearrange readily with silicon esters, it seemed logical to investigate the conditions under which silicon alkyls will rearrange with silicon esters.

TABLE VI

RESULTS OF EXPERIMENT F4

Peak	*Identity*	*Relative retention ratio (w.r.t. Et_2O)*
a	$SnMe_4$	1.30
b	Unknown	1.50
c	Unknown	1.76
d	Unknown	2.16
e	Unknown	2.34
f	$Si(OEt)_4$	3.08

System G: $Si(OEt)_4 + SiPr_4$

Experiment G1. Reactants were heated at atmospheric pressure in the absence of a catalyst at 120°. Samples examined at intervals by GLC (data as for E3) showed no rearrangement up to 10 days, at which time the reaction mixture became solid.

Experiment G2. Reactants were heated together at atmospheric pressure with a trace of $SiCl_4$ at 120°. GLC analysis showed that some reaction occurs, but that after 24 h further heating causes decomposition of the reaction products. A trace of the analysis of Experiment G2 after 24 h is shown in Fig. 7. (See also Table VII.)

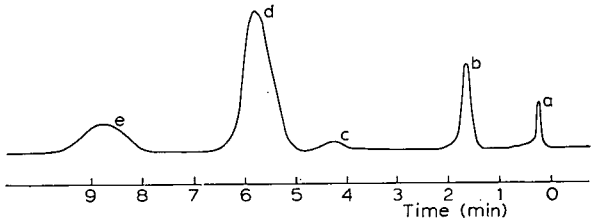


Fig. 7. Experiment G2 at 24 h. Reaction between $Si(OEt)_4$ and $Si(n\text{-}Pr)_4$. (a) = Diethyl ether, (b) = $Si(OEt)_4$, (c) = unknown, (d) = $Si(n\text{-}Pr)_4$, (e) = unknown.

TABLE VII

RESULTS OF EXPERIMENT G2

Peak	*Identity*	*Relative retention ratio*
a	Et_2O	1.00
b	$Si(OEt)_4$	3.08
c	Unknown	6.21
d	$SiPr_4$	9.21
e	Unknown	13.9

Experiment G3 and G4. Reactants were heated at atmospheric pressure with a trace of $AlCl_3$ at 120° (G3) and at 170° (G4). In neither case was any rearrangement revealed by GLC, and in both cases the reaction mixture became solid within 5 days, due once more to decomposition of $Si(OEt)_4$.

Since system G was a relative failure, the silicon alkyl most like ethyl silicate was chosen for the next series.

System H: $Si(OEt)_4 + SiEt_4$

Experiment H1. Reactants were heated together at atmospheric pressure with a trace of $SiCl_4$ at 120°. GLC analysis showed that after 5 days no rearrangement had occurred. The presence of Et_2O was observed, however, and once again the $Si(OEt)_4$ peak was much reduced in size.

Column temperature: 100°; phase: 25% silicone oil/celite 60–85;
p_i/p_o (N_2) = 1.6.

TABLE VIII

REARRANGEMENTS INVOLVING ESTERS OF SILICON AND PHOSPHORUS

Reactants	*Pressure*	*Catalyst*	*Temperature (°C)*	*Time*	*Rearrangement*
$OP(OMe)_3 + OP(OEt)_3$	A[b]	$AlCl_3$	120	18 hours	Nil
$OP(OMe)_3 + OP(OEt)_3$	S.T.[a]	$NaOCH_3$	120	7 days	100%
$Si(OMe)_4 + Si(OEt)_4$	S.T.	None	120	2 days	100%
$OP(OEt)_3 + Si(OMe)_4$	S.T.	$NaOCH_3$	120	7 days	Nil
$OP(OEt)_3 + Si(OMe)_4$	S.T.	None	120	14 days	Slight
$OP(OEt)_3 + Si(OMe)_4$	S.T.	None	180	14 days	Decomposition
$OP(OEt)_3 + Si(OMe)_4$	A	$SiCl_4$	120	20 days	Decomposition
$OP(OEt)_3 + Si(OMe)_4$	S.T.	$SiCl_4$	120	14 days	Nil, but Et_2O formed
$OP(OMe)_3 + Si(OEt)_4$	S.T.	$NaOCH_3$	120	7 days	Nil
$OP(OMe)_3 + Si(OEt)_4$	S.T.	None	120	7 days	Nil
$OP(OMe)_3 + Si(OEt)_4$	A	$SiCl_4$	120	10 days	Nil, but Et_2O formed

[a] S.T. = Reactants in sealed tubes.
[b] A = Reactants heated at atmospheric pressure.

TABLE IX

REARRANGEMENTS INVOLVING OTHER CENTRAL ATOMS

Reactants	*Pressure*	*Catalyst*	*Temperature (°C)*	*Time*	*Rearrangement*
$SnMe_4 + OP(OEt)_3$	S.T.[a]	None	120	8 weeks	Nil
$SnMe_4 + OP(OEt)_3$	S.T.	None	150	8 weeks	Nil, Et_2O formed
$SnMe_4 + OP(OEt)_3$	S.T.	None	180	14 days	Extensive
$SnMe_4 + OP(OEt)_3$	S.T.	$AlCl_3$	180	few hours	Decomposition
$SnMe_4 + OP(OEt)_3$	S.T.	$AlCl_3$	120	24 hours	Decomposition
$SnMe_4 + Si(OEt)_4$	S.T.	None	120	10 weeks	Nil
$SnMe_4 + Si(OEt)_4$	A[b]	None	120	30 days	Nil
$SnMe_4 + Si(OEt)_4$	A	$AlCl_3$	120	10 days	$Si(OEt)_4$ decomposes
$SnMe_4 + Si(OEt)_4$	A	$SiCl_4$	120	14 days	$Si(OEt)_4$ decomposes
$Si(OEt)_4 + Si(Pr)_4$	A	None	120	10 days	Nil
$Si(OEt)_4 + Si(Pr)_4$	A	$SiCl_4$	120	24 hours	Nil, Et_2O formed
$Si(OEt)_4 + Si(Pr)_4$	A	$AlCl_3$	120	5 days	Decomposition
$Si(OEt)_4 + Si(Pr)_4$	A	$AlCl_3$	170	5 days	Decomposition
$Si(OEt)_4 + SiEt_4$	A	$SiCl_4$	120	5 days	Nil, Et_2O formed
$SiEt_4 + SiPr_4$	A	$SiCl_4$	120	40 min	100%

The conclusions that can be drawn from Experiment H1 are:

Either (a) no rearrangement has occurred,

or (b) rearrangement takes place by fission of the O–Et bond.

In case (b), no intermediate mixed ethyl–ethoxy silanes will be observed.

In conclusion, it was found that under the same experimental conditions as Experiment H1, two alkylsilanes ($SiEt_4$ + $SiPr_4$) undergo complete rearrangement in about 40 min.

RESULTS

The results obtained from the experiments described in this paper are summarised in Tables VIII and IX.

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GAS CHROMATOGRAPHIC METHODS FOR MIXTURES OF INORGANIC GASES AND C_1–C_2 HYDROCARBONS

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SUMMARY

Five gas chromatographic methods have been developed whereby gas mixtures containing H_2, A (or O_2), N_2, CO, CO_2, CH_4, and C_2H_6 can be analyzed on various lengths and combinations of three kinds of gas–solid adsorption columns. The adsorbents employed are molecular sieve 5A, silica gel and activated charcoal. Volume of gas mixture injected into the column is fixed at 0.5 ml, and therefore, volume percent of each component can be directly determined from its calibration curves. The calibration curves are constructed by plotting peak area versus percentage of the component in 0.5 ml sample of a series of standard mixtures with attenuation of the instrument as the parameter. When greater accuracy is desired, a normalization method is used.

Preparation of gas samples for chromatography, including sampling and conditioning, are also presented and discussed in detail.

All five methods are simple, rapid, and accurate. They have been satisfactorily applied to gas samples from coal gasification. Two of them are also applicable for on-stream analyses.

INTRODUCTION

The gas generator research project at Bituminous Coal Research, Inc., called for development of rapid and accurate methods for analyzing gaseous products containing inorganic gases and C_1–C_2 hydrocarbons. Due to its versatility and rapidity, gas chromatography was extensively employed.

Gas chromatography using thermal conductivity detection has been applied to gas mixtures of fixed gases and light hydrocarbons by many authors. For a review of the literature, see also ref. 5. Generally, two or more columns—a molecular sieve column with either a gas–solid adsorption or a gas–liquid partition column—are used for a complete analysis. CVEJANOVICH[1] separated mixtures of C_1–C_5 hydrocarbons and inorganic gases on three columns, namely a squalane on chromosorb, an adiponitrile on chromosorb and a molecular sieve 5A. The technique is rather involved and the arrangement of the columns is complicated. SWINNERTON and co-workers[2] employed, in series, a hexamethyl-phosphoramide on Columpak and a molecular sieve 13X, to

determine dissolved CO_2, O_2, N_2, CH_4, and CO in aqueous solutions. Later MANKA[3] also used, in series, a silica gel and a molecular sieve 13X, to analyze the same components in gas samples. In the latter case, only one detector was employed; therefore, switching polarity of the detector was necessary. These methods all have their merits and are good for their specific applications.

In our laboratory, a large number of gas samples either from coal gasification studies or from coal pyrolysis studies were to be analyzed. The components were: major, H_2, A, N_2, CO, CO_2, and CH_4, minor, C_2H_6, and traces, C_2H_4 plus some sulfides, which were undesirable impurities. Argon was present only in the gasification samples and was purposely added to the gasifier as a reference for making material balance in coal gasification studies[4]. To meet our need, the analytical procedures had to be highly accurate, rapid, and simple. After examining and testing the existing procedures, none of them met all the criteria. To suit our various purposes, five methods, using silica gel, activated charcoal, and molecular sieve 5A columns, were developed. Of the five, two (methods A and B) have become routine procedures to handle daily samples in the laboratory, another two (methods D and E) have been satisfactorily applied to our own on-stream analysis, and only method C appears to have limited usage.

This paper describes the five methods, their operating conditions, method of determining component concentrations, precision, and sample preparation.

EXPERIMENTAL

Gas chromatographs and columns

Two F & M gas chromatographs, Model 720 and Model 700-231, were used. Both were equipped with thermal conductivity detectors, dual columns, and Honeywell 1-mV recorder with automatic disc integrators for peak areas. The Model 720 was provided with a single gas sampling valve and the 700-231 with two valves, one for each column.

Helium was chosen as the carrier gas. As pointed out in the literature[5], a suitable mobile phase for the thermoconductivity detector is helium or hydrogen with a slight preference of the latter. However, in our case, hydrogen was a major component of the sample, and helium was, therefore, the natural choice.

Columns were all 0.25 in. O.D. aluminum tubing packed in this laboratory with one of the three packing materials, namely silica gel (30–60 mesh), molecular sieve 5A (30–60 mesh), or activated charcoal (30–60 mesh). The materials were purchased from F & M Scientific Company. Packing material per foot of column was 5 g for silica gel, 4 g for molecular sieve, and 2.5 g for charcoal. The columns were packed by a combination of vacuum and vibration techniques. The newly packed columns and exhausted columns were activated with helium. The activation was accomplished for molecular sieve and charcoal columns at 350° for 3 hours and for silica gel at 160° for 4 hours.

Column design and operating conditions

Design of column and establishment of operating conditions were partially guided by the principles discussed in the literature[5–7] and partially based on experience for finer adjustments. After extensive experimentation, satisfactory combinations were achieved for various gas mixtures. The final results are shown in Table I.

TABLE I

DESCRIPTION OF FIVE GC METHODS FOR MIXTURES OF INORGANIC GASES AND SOME LIGHT HYDROCARBONS

	A	*B*	*C*	*D*	*E*
Column	6 ft. (or 3 ft.) Molecular Sieve 5A / 3 ft. Silica gel (Independent)	12 ft. Silica gel	3 ft. Silica gel in series with 12 ft. Molecular Sieve 5A	3 ft. Molecular Sieve 5A	2 ft. Carbon column
Model of gas chromatograph	F & M 700-231	F & M 720	F & M 720	F & M 720	F & M 720
Elution order of components	H_2, A(O_2), N_2, CH_4, CO (M.S.); composite, C_2H_6, CO_2, C_2H_4 (S.g.)	H_2, A(O_2) + N_2, CO, CH_4, C_2H_6, CO_2, C_2H_4, C_3H_8	H_2, A(O_2), N_2, (all from M.S.) CO_2 (S.g.), CH_4 (M.S.) CO (M.S.)	H_2, A(O_2), N_2, CH_4 CO	H_2, composite (A, O_2, N_2, CO) CH_4, CO_2
Operating conditions					
Column temperature (°C)	50	50	50	50	80
Detector temperature (°C)	140	120	120	120	120
Bridge current (mA)	200	190	190	190	190
Injection port temperature (°C)	90	90	90	110	110
Helium pressure (p.s.i.)	30	30	30	30	30

Helium flow rate at exit of the column (ml/min)	50	50	60	50	50
Sampling loop (ml)	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$
Remarks	(a) CO_2 removed from sample prior to admission to molecular sieve column. (b) A and O_2 can be determined by CHANG's differential method[8]. (c) Sample may be stripped of CO_2 prior to admission to silica gel column when determination of minute quantity of C_2H_4 is desired.	(a) O_2 and (A + N_2) as a group can be determined by the differential method[8].	(a) Two columns connected with a 10 in. × $^1/_8$ in. teflon tubing; switching detector polarity is necessary. (b) A small CO_2 trap is inserted between the connecting tubing and the molecular sieve column. (c) A and O_2 can be individually determined[8].	(a) CO_2 removed from sample prior to admission to column. (b) Good for on-stream application.	(a) Good for on-stream application.

Preparation of gas samples

Sampling

Depending on whether the analysis was to be made in the laboratory or on-stream in the pilot plant, two different means of sampling were used. For laboratory use, batch samples were collected in either a glass sampler or a metal sampler. For on-stream analysis, the gas was introduced directly into the instrument.

The glass sampling system for low gas pressure as devised in this laboratory is shown schematically in Fig. 1. (T) is a 500 ml glass sampling tube connected to a manifold (M). Each tube was filled with a confining liquid containing a saturated solution of Na_2SO_4 acidified with H_2SO_4 to 20% concentration. Methyl orange was added to the liquid to indicate the acidity of the solution. Basic constituents, such as NH_3, reacted with the acid and stayed in the liquid while all acidic components, such as CO_2 and H_2S, as well as neutrals, remained in the gas phase. (C) is a leveling bulb serving as a reservoir for the confining liquid. This system was satisfactorily used with gas line pressures from 10 in. to 40 in. water. Time for each collection was manually adjusted from a few seconds to a few minutes at a constant flow rate as desired. When samples were collected by this system, CO_2 could be determined by other wet methods for higher accuracy, such as the standard Orsat absorption method[9] used in this laboratory. This point will be discussed later in the paper.

For higher gas line pressure and larger volume of the gas sample, an all stainless steel batch sampling system was devised. It consisted of several 1500 ml-cylinders, each equipped with a packless valve and $^1/_8$ in. tubing fittings. The cylinders were connected to short parallel pieces ($^3/_4$ in. long) of $^1/_8$ in.-O.D. tubing welded on a $^1/_4$ in.-O.D. tubing used as manifold. The inlet end of the manifold was equipped with a single-stage pressure regulator in series with an MSA filter cartridge; the outlet end with a control valve. The system was connected to the gas line at the filter cartridge and

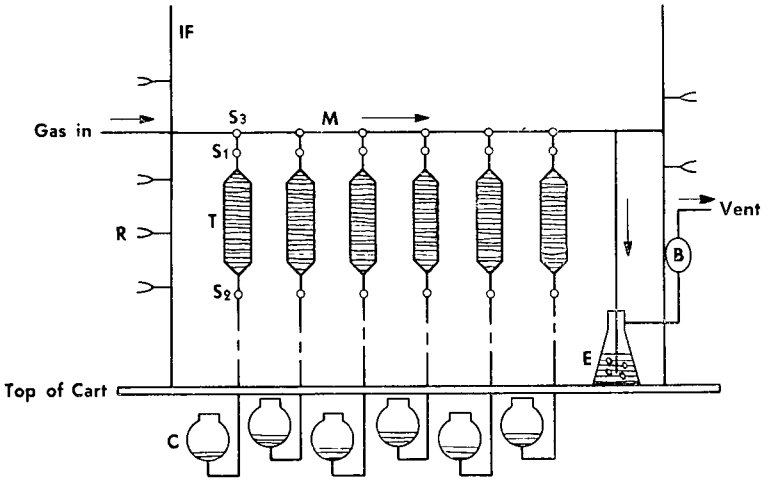

Fig. 1. Batch sampling system on cart. T = Sampling tube; S_1, S_2 = two-way stopcock on sampling tube; S_3 = three-way stopcock on manifold; M = manifold; IF = iron frame; C = confining liquid reservoir connected to sampling tube by long tygon tubing; E = Erlenmeyer flask containing water to indicate gas flow; B = double action bulb; R = iron ring for reservoir.

evacuated prior to sampling. After evacuation, all cylinders were kept under vacuum by closing their valves. The gas to be sampled was let in at the cartridge through the manifold and vented to atmosphere at a regulated pressure of 1 to 2 p.s.i. for purging. The control valve at the outlet was then closed. One of the cylinder valves was opened to sample the gas. The pressure of the gas was gradually increased at approximately constant rate to 20 p.s.i. within a few seconds to a few minutes as desired. At the end of the sampling period, the cylinder valve was closed and the outlet control valve opened again. The pressure was returned to 1 to 2 p.s.i. for purging. This procedure was repeated for the next sampling. The sample so collected represented an average product within the sampling period.

Conditioning of samples

Regardless of the sampling devices, the sample must be conditioned prior to admission to the gas chromatograph. A glass purification train, shown in Fig. 2, was inserted between the sample and the inlet of the chromatograph. Absorber (A) of 20 ml-capacity contained 10 to 15 ml $CdCl_2$ solution to trap sulfides[10]. Refrigerator (B) was made of a 2 mm I.D. coiled glass tubing attached to a 6 mm tubing and was placed in a Dewar flask packed with cracked ice. Drying tube (C) was a 3 in. $\times$ $^1/_4$ in. I.D. tygon tubing filled with indicating drierite (10 to 20 mesh). The total hold-up volume of the train was 30 ml. For the analysis of batch samples, the train and the sampling loop must be purged thoroughly with the sample prior to injection. For this reason, a minimum of 150 ml gas was needed for each analysis.

For on-stream gas chromatography, a much larger sulfide trap (50 ml), or two traps in series, and a longer drying tube (C) were needed. Exhausted traps could be

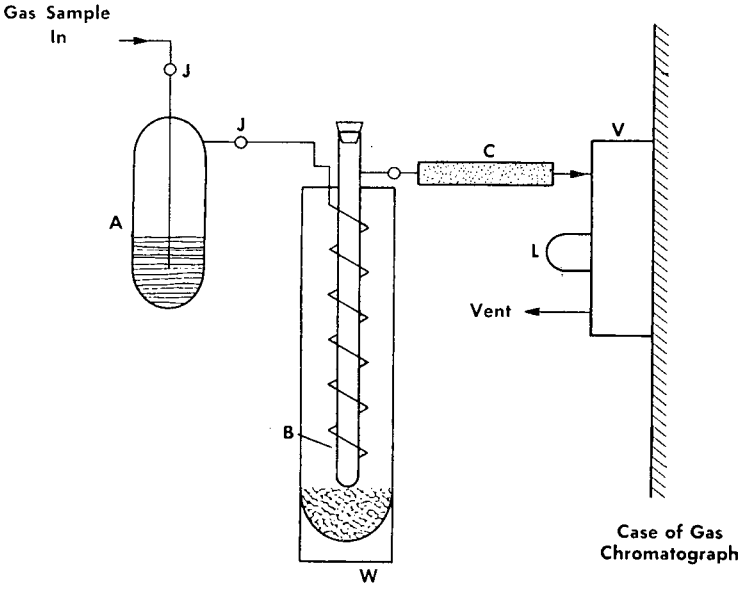

Fig. 2. Purification train for gas samples. A = Sulfide absorber; B = refrigeration tube; C = drying tube; V = sampling valve; L = sampling loop; J = ball joints; W = Dewar flask. Connecting tubing: 2 mm I.D. heavy wall capillary.

replaced with fresh ones between injections. The proper flow rate for gas flowing through the train was found to be about 100 ml/min.

Removal of CO_2 in samples

Presence of CO_2 in samples presented a problem on the molecular sieve column in methods (A), (C), and (D). CO_2 was rather strongly adsorbed on this column and eluted very slowly at 50°. This caused delay in readying the column for other injections. Removal of CO_2 from the sample eliminated the problem. A cartridge made of a 4 in. $\times$ $^3/_{16}$ in. I.D. glass tubing filled with Indicarb (10 to 20-mesh) was used for this purpose. The ends of the cartridge were loosely plugged with glass wool and tightly fitted with $^1/_8$ in.-holed rubber plugs. Stainless steel tubing of $^1/_8$ in. O.D. connects the cartridge between the sampling valve and the inlet of the column. Such a cartridge may be similarly employed in method (E) should on-stream measurement be limited to H_2 and CH_4 only.

Determination of concentration of each component

For each of the five methods, a family of calibration curves was established for each component by chromatographing standard mixtures of increasing concentration at various instrument attenuations. The curves were constructed by plotting integrated peak area directly *versus* the percentage of component in 0.5 ml of standard at ambient conditions with attenuation as the parameter. When an unknown was chromatographed under the standard operating conditions, the percentage of a component was determined from its peak area on the calibration curve.

However, if the total percentages of components in the unknown differed from 100, a normalization method was used to improve the accuracy of the result.

When the 500 ml-glass sampler with confining liquid was used, it was found that appreciable amounts of CO_2 would dissolve in the liquid if prolonged contact of the two was allowed. This condition was encountered when the gas in the sampler was

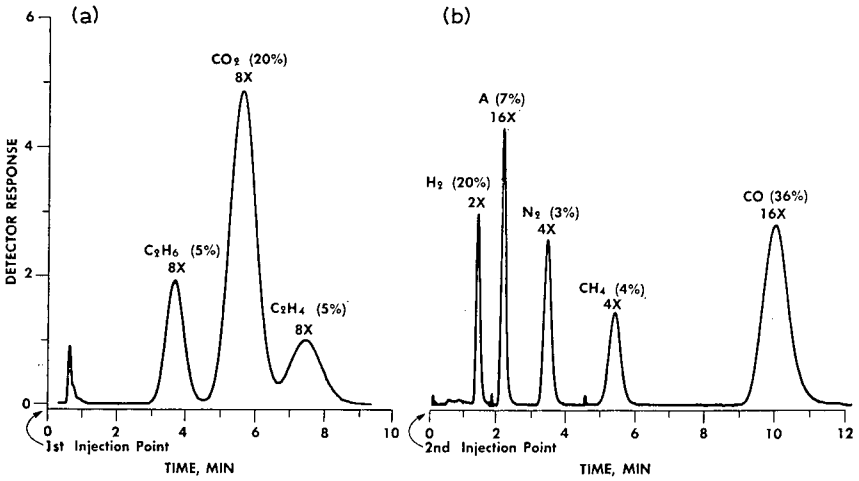

Fig. 3. Chromatograms produced by method A. (a) 3 ft. silica gel at 50°. (b) 6 ft. molecular sieve 5A at 50°.

repeatedly expelled by the incoming liquid. As discussed later, loss of CO_2 could amount to a few percent depending on its partial pressure. To eliminate this trouble, CO_2 was determined by the standard Orsat method immediately after sampling. The remaining components were determined by a suitable GC method. For this case, the calculation is as follows:

$$f_n = \frac{100 - CO_2\% \text{ (from Orsat)}}{(H_2{}^{R}\% + N_2{}^{R}\% + CH_4{}^{R}\% + ...)}$$

$$H_2\% = f_n\,(H_2{}^{R}\%)$$
$$N_2\% = f_n\,(N_2{}^{R}\%)$$
$$CH_4\% = f_n\,(CH_4{}^{R}\%)$$

where:

f_n = normalization factor,

$H_2{}^{R}\%$, $N_2{}^{R}\%$, $CH_4{}^{R}\%$..... = read out % of H_2, N_2, CH_4 ....., from their calibration curves.

$H_2\%$, $N_2\%$, $CH_4\%$ ..... = normalized % of H_2, N_2, CH_4 .....

For on-stream analysis, using methods (D) and (E), only a few important components were generally determined. Their percentages were found directly from the peak areas on the calibration curves.

RESULTS AND PRECISION OF THE METHODS

Chromatograms produced by each of the five methods are shown in Figs. 3, 4, 5, and 6. Relative retention times referring to N_2 for the components are presented in Table II.

The time requirements for the five methods are as follows: method (A), 30 min

TABLE II

RELATIVE RETENTION TIME (R_t) OF GAS COMPONENTS ON DIFFERENT COLUMNS

Method	*Column*	R_t*							
		H_2	$A(O_2)$	N_2	CH_4	C_2H_6	CO	CO_2	C_2H_4
(A)	6 ft. molecular sieve 5A	0.31	0.55	1* (2.9 min)	1.66	16.80	3.31	—	—
	3 ft. silica gel	0.50	1.0	1 (0.6 min)	1.0	6.30	1.0	10.0	13.00
(B)	12 ft. silica gel	0.65	1.0	1 (2.1 min)	1.55	7.45	1.20	10.70	14.1
(C)	3 ft. silica gel in series with 12 ft. molecular sieve	0.09	0.14	0.14	0.20	—	0.14	1.52	—
		0.35	0.67	1 (6.4 min)	1.95	—	2.50	—	—
(D)	3 ft. molecular sieve 5A	0.36	0.64	1 (1.4 min)	1.71	21.5	2.57	—	—
(E)	2 ft. carbon	0.57	1.00	1 (0.7 min)	2.14	—	1.00	4.86	—

* Reference: N_2.

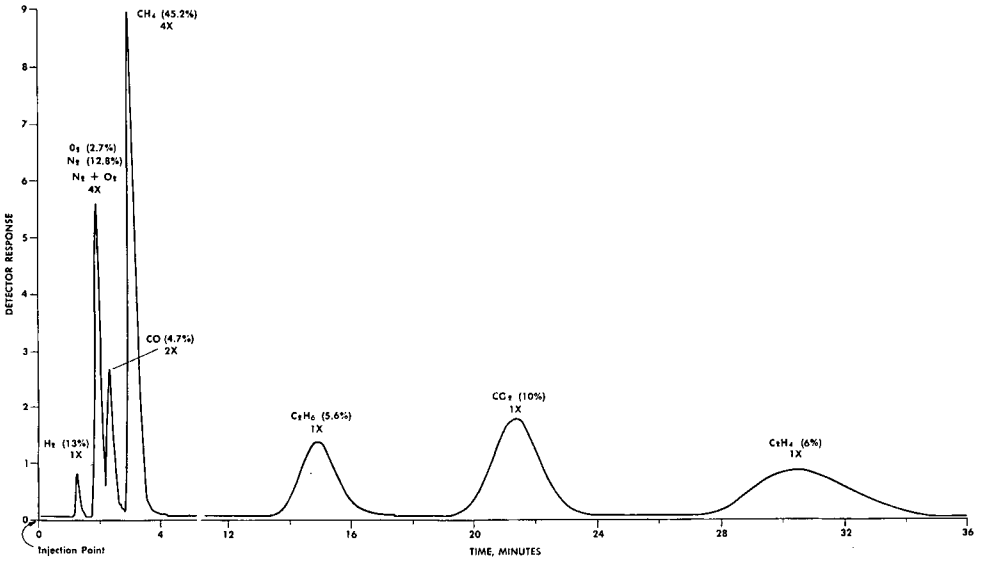

Fig. 4. Chromatogram produced by method B.

to C_2H_6; method (B), 35 min to C_2H_4; method (C), 18 min to CH_4; method (D), 4 min for analyzing H_2, A(O_2), N_2, CH_4, and CO; method (E), 4 min for determining H_2, CH_4 and CO_2, or only 2 min for H_2 and CH_4.

Generally speaking, precision of any GC method depends on several factors,

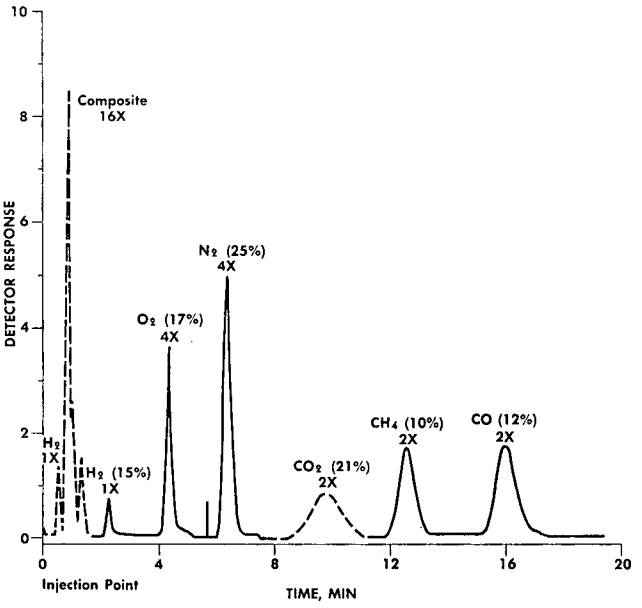

Fig. 5. Chromatogram produced by method C. 3 ft. silica gel (- - - -) in series with 12 ft. molecular sieve 5A (———) at 50°.

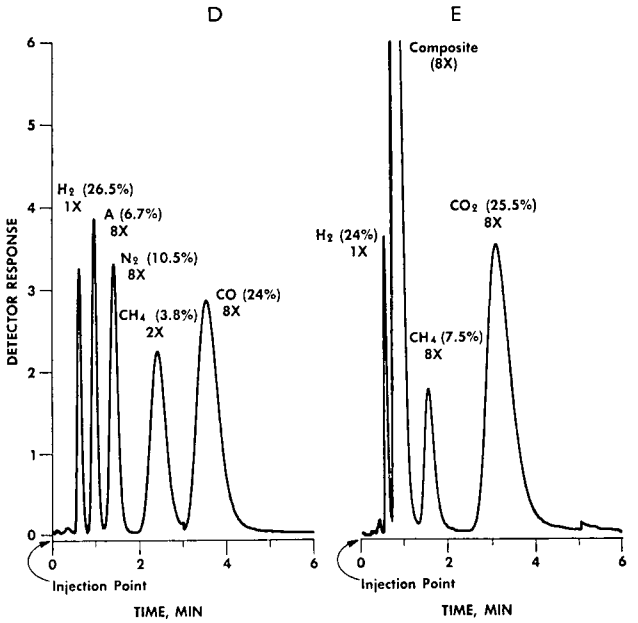

Fig. 6. Chromatograms produced by method D and method E. (Result of on-stream application.) Method D: 3 ft. molecular sieve 5A at 50°. Method E: 2 ft. charcoal at 80°.

namely, sampling and injection techniques, chromatograph and recorder performances, and ambient conditions. For method (A), the F & M 700-231 gas chromatograph was used. The precision of this method using this instrument for analyzing H_2, A, N_2, CH_4, CO and C_2H_6 plus CO_2 by the Orsat determination is expressed as standard deviations[11] as shown in Table III. These deviations were calculated from the results of replicate analyses of a sample from coal gasification. For applying methods (B), (C), (D), and (E), the F & M 720 gas chromatograph was employed. It is felt sufficient to present the precision data from one method for this instrument. As shown in Table IV, the precision

TABLE III

REPLICATE ANALYSES OF A GAS SAMPLE BY METHOD (A)

Run No.	*Volume (%)*						
	CO_2*	C_2H_6	H_2	A	N_2	CH_4	CO
1	18.6	1.8	24.8	11.9	1.9	10.5	30.5
2	18.5	1.9	24.6	11.9	2.0	10.7	30.4
3	18.5	1.8	24.8	11.9	1.9	10.7	30.4
4	18.3	1.3	25.9	11.8	1.8	10.7	30.2
5	18.6	1.5	25.9	11.8	1.9	10.2	30.1
6	18.5	1.5	25.1	11.7	1.9	10.8	30.5
Mean	18.50	1.63	25.18	11.84	1.90	10.60	30.35
Std. dev. (σ)	0.110	0.233	0.577	0.082	0.100	0.219	0.164

* CO_2 by Orsat method.

TABLE IV

REPLICATE ANALYSES OF A KNOWN MIXTURE BY METHOD (B)

Run No.	*Volume (%)*				
	H_2	CO	CO_2	CH_4	C_2H_6
1	6.2	0.80	1.0	2.50	0.75
2	5.9	0.85	1.05	2.50	0.95
3	5.8	0.85	1.20	2.60	0.80
4	6.0	0.80	1.00	2.50	0.95
5	5.7	0.85	1.00	2.80	0.90
Mean	5.9	0.83	1.05	2.60	0.87
% Present*	6.06	0.80	1.10	2.44	0.85
Std. dev. (σ)	0.193	0.042	0.141	0.042	0.091

* Balance of the mixture was helium.

of method (B) and the instrument is expressed as standard deviations determined by replicate analyses of a simulated gas sample from the coal pyrolysis. These deviations are considered to be small for components at such low concentrations.

DISCUSSION

As described in the experimental section, two sampling systems were used in this work to obtain batch samples. One was an all glass unit for low pressure gas, and the other an all stainless steel unit for high pressure gas. The former system used a confining liquid which absorbs appreciable amounts of CO_2 when its partial pressure was high.

When CO_2 was determined by gas chromatography, the purification train and the sampling loop were slowly and thoroughly purged with a large volume of the sample, which was gradually forced out by admitting the confining liquid into the sampling tube. Before an injection of the sample could be made to the column, CO_2 in the gas sample was gradually absorbed by the liquid tending to establish an equilibrium between the two phases. As a result, CO_2 concentration in the gas phase became less as time passed and the peaks produced by consecutive injections of the sample became smaller and smaller. It was found that for samples having 25% CO_2, the area difference of the highest and the lowest peaks reached 2 to 3%, and for those with 35% CO_2, 3 to 4%. To correct this error, CO_2 was determined by the Orsat method prior to gas chromatography of the sample. Immediately after sampling, the tube was full of gas under a pressure slightly higher than 1 atm. Less than 10 ml of the confining liquid was left inside, and this small amount of liquid was already saturated with CO_2. When the first portions of the sample were taken out for Orsat CO_2 determination, the result would closely represent the true concentration of this component. It was also found that for samples with less than 10% CO_2, the loss to confining liquid was not large enough to cause significant error. Therefore, Orsat CO_2 determination was not applied to samples having CO_2 less than 5%. This absorption of gas by the confining liquid was observed with CO_2 but not with other components.

By using the metal sampling system, loss of CO_2 was avoided. Besides, the basic constituents, *i.e.*, NH_3 and pyridine homologs, if present, would still remain in the sample. The large quantity of an intact sample so collected could supply the need for many other purposes. On the other hand, the cost of the metal system was high and it could not be assembled in the laboratory as easily as the glass unit.

As described in the section *Determination of concentration*, calibration curves for all five methods were established from areas produced by 0.5 ml standard mixtures measured at ambient conditions. Theoretically, gas sampled at ambient conditions must be corrected to standard temperature and pressure; however, we found that the correction was unnecessary. In our locality, barometric pressure recorded for a period of four months was 730 $\pm$ 5 mm and room temperature in the air-conditioned laboratory was 26° $\pm$ 2°. The error introduced to the volume by these variations was found insignificant. To simplify the procedure, no correction of temperature and pressure was made for the 0.5 ml volume of the standard mixtures during calibration. For unknown samples, it is noted that the procedure of normalization also tends to cancel out the effect of pressure and temperature.

In method (A), as shown in Table I, either a 6 ft. or a 3 ft. molecular sieve column completely separates H_2, A, N_2, CH_4, and CO. However, the longer column was preferred because the resolution values between adjacent peaks were greater than on the shorter column; thus providing a safety factor against wide variations in the molar ratios of adjacent components.

Also in method (A), the 3 ft. silica gel column did not completely resolve CO_2 and C_2H_4 at a molar ratio (C_2H_4 to CO_2) of 0.25 as shown in Fig. 3, and a 10 ft. column was needed for complete separation. But on this longer column, retention times of these two components were too long. It was impractical to couple the 10 ft. column with the 6 ft. molecular sieve for routine use. Fortunately the majority of the gas samples encountered contained no C_2H_4. Whenever samples containing minute amounts of C_2H_4 were determined on the 3 ft. silica gel, the analysis was made by removing CO_2 from the sample with an "Indicarb" cartridge installed between the column inlet and the sampling valve. In this manner, C_2H_4 appeared as a small individual peak. Therefore, for speed, the 3 ft. silica gel column was employed in method (A).

The precision of methods (A) and (B) shown in Tables III and IV is high; these two methods, therefore, have become routine procedures for use in our laboratory.

Methods (D) and (E) were generally used for on-stream analysis of a few important components. Since normalization could not be accomplished, the precision was sometimes slightly lower than that of methods (A) and (B). For operation control purposes, methods (D) and (E) were found satisfactory.

Method (C), which uses a 3 ft. silica gel column in series with a 12 ft. molecular sieve, is similar to the method developed by MANKA[3]. In method (C), as shown in Fig. 5, CO_2 from the silica gel column is eluted between N_2 and CH_4, both from the molecular sieve. But in MANKA's method, elution of CO_2 from the silica gel can be adjusted either ahead of or behind the other components emerged from the molecular sieve column. It may be true that by varying the length of the connecting tubing between the two columns and the operating conditions of the gas chromatograph, elution of CO_2 could be spaced anywhere as desired; however, spacing CO_2 at the beginning or in the middle of the chromatogram was found undesirable. When the

concentration of CO_2 and its adjacent components differed greatly, the elution curve for these two would be distorted somewhat and quantitative estimation could not be made accurately. For two closely eluted peaks, as CO_2 and CH_4 in this case, resolution is affected by their molar ratio[5]. In gas–solid chromatography, retention time and peak broadening usually increase with concentration and thus influence the difference of retention time between the two peaks. The resolution will deteriorate with decreasing molar ratio of CH_4 to CO_2 and vice versa. For example, from our experience, when a high percent CO_2 was eluted between N_2 and low percent CH_4, the small CH_4 peak was only partially shown or entirely lost. This is due to the fact that the last part of the major CO_2 peak at a lower instrument attenuation overlapped with the small CH_4 peak which was partially or entirely cancelled while reversing the detector polarity. To avoid this situation, CO_2 elution must be spaced at the end, far away from the last eluate from the molecular sieve. When the molar ratio of CH_4 to CO_2 was in the range of 0.75 to 0.25 with $CO_2 < 25\%$ in a 0.5 ml sample, the elution pattern produced by method (C) was not distorted.

In conclusion, all five methods as described above have been satisfactorily applied to coal gasification samples in our laboratory. It is believed that methods (A), (B), (D), and (E) can be equally well applied to similar gas samples from other sources. In addition, methods (D) and (E) are extremely useful for on-stream application in gas-making processes.

ACKNOWLEDGEMENT

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CHROM. 3620

CHROMATOGRAPHY OF THE ISOMERIC METHYLENE-INTERRUPTED METHYL *CIS*, *CIS*-OCTADECADIENOATES

2. GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

The equivalent chain lengths on gas–liquid chromatography of the isomeric methylene-interrupted methyl *cis*, *cis*-octadecadienoates (2,5- to 14,17-) were determined on eight different liquid phases, two of which were in capillary columns. The equivalent chain length values increased with distance of the double bonds from the carboxyl group but the values for the 3,6- and 13,16-isomers on polar columns or the 13,16-isomer alone on the non-polar column were much greater than those of the adjacent isomers. Isomers differing by 0.04 in equivalent chain length could be separated on the Apiezon L capillary column and those differing by 0.08 in equivalent chain length on the neopentyl glycol succinate capillary column.

INTRODUCTION

Preliminary or tentative identification of unknown unsaturated fatty acids in lipid samples by their relative retention times on various gas–liquid chromatography (GLC) columns is common practice in many laboratories. This has been considerably facilitated by the introduction of the concept of carbon numbers (WOODFORD AND VAN GENT)[1] or equivalent chain lengths (ECLs) (MIWA, MIKOLAJCZAK, EARLE AND WOLFF)[2] for presenting retention data. Recent reviews[3,4] describe the merits of the systems in fatty acid analysis. ECLs for large numbers of fatty acids on many GLC liquid phases are available in the literature and there have been many attempts, particularly in the laboratories of ACKMAN[5,6] and HOLMAN[4,7], to correlate these values with the structures of the acids. The number of different fatty acids available from natural sources is limited, however, and studies with complete series of model compounds are necessary for more meaningful conclusions to be drawn. GUNSTONE, ISMAIL AND LIE KEN JIE[8] have recently recorded retention data for the complete series of isomeric methyl *cis* and *trans* octadecenoates. A similar study of the retention data and separations achieved of the isomeric methylene-interrupted methyl *cis-cis-*

* Part 1. see ref. 19.

octadecadienoates (*i.e.* methyl *cis*-2-, *cis*-5- to *cis*-14,17-octadecadienoate[9]) is now described.

EXPERIMENTAL

The columns used with the appropriate operating conditions are detailed in Table I. Capillary columns were used in an F11 gas chromatograph (Perkin-Elmer Ltd, Beaconsfield) and packed columns in a Pye 104 (W.G. Pye Ltd, Cambridge). Both instruments were equipped with flame ionization detectors and nitrogen was the carrier gas. Solid supports were acid-washed and silanised. C_{14}, C_{16} and C_{17} or C_{18}

TABLE I

CONDITIONS OF GAS–LIQUID CHROMATOGRAPHY WITH EACH OF THE LIQUID PHASES

Liquid phase	*Content (%)*	*Support*	*Mesh*	*Column dimensions*	*Flow rate (ml/min)*	*Temp.*
ApL (1)*				50 m × $\frac{1}{4}$ mm	*ca.* 2	220
NPGS*				50 m × $\frac{1}{4}$ mm	*ca.* 2	190
ApL (2)	5	Gas Chrom Z	70–80	5 ft. × $\frac{1}{4}$ in.	50	200
Carbowax 20 M-terephthalic acid	5	Chromosorb G	80–100	5 ft. × $\frac{1}{4}$ in.	50	200
PEGA	15	Gas Chrom Z	70–80	5 ft. × $\frac{1}{4}$ in.	50	190
DEGS	20	Gas Chrom Z	70–80	5 ft. × $\frac{1}{4}$ in.	50	180
EGS	20	Chromosorb W	100–120	7 ft. × $\frac{1}{4}$ in.	50	180
EGS–2% H_3PO_4	20	Gas Chrom P	80–100	7 ft. × $\frac{1}{4}$ in.	50	180

* Capillary columns.

saturated methyl esters were used as internal standards. The relative retention times for each of the esters were determined on the eight different liquid phases and are presented as ECLs. All columns were freshly packed since it is well documented that ECL values can alter as columns age. The capillary columns were purchased precoated (Perkin-Elmer Ltd, Beaconsfield).

RESULTS AND DISCUSSION

The liquid phases chosen were those that are most commonly in use in lipid laboratories. Apiezon L (ApL) is non-polar and unsaturated esters are eluted before the corresponding saturated esters. Polyethylene glycol adipate (PEGA), diethylene glycol succinate (DEGS) and ethylene glycol succinate (EGS) are the commonest polar polyester liquid phases in use. EGS with added phosphoric acid is reported to be more stable and to give better separations than EGS alone[10]. Neopentyl glycol succinate (NPGS) is commercially available in capillary (open-tubular or Golay) columns. Carbowax 20 M terephthalic acid is commonly used for the chromatography of free acids. The ECL values obtained for each isomer on each of the eight columns are listed in Table II. Those from three representative columns are illustrated graphically in Fig. 1.

On all the polar liquid phases, the ECL values of the isomers increase with

TABLE II

EQUIVALENT CHAIN LENGTHS OF THE ISOMERS ON POLAR AND NON-POLAR COLUMNS

Isomer	*Liquid phase*							
	ApL (1)[a]	*NPGS*	*ApL (2)*[b]	*Carbo-wax*	*PEGA*	*DEGS*	*EGS*	*EGS–H_3PO_4*
Methyl 2,5-octadecadienoate	17.68[c]	18.14	17.64	18.25	18.37	18.64	18.65	18.72
Methyl 3,6-octadecadienoate	17.62	18.65	17.57	18.67	18.94	19.36	19.54	19.68
Methyl 4,7-octadecadienoate	17.47	18.36	17.42	18.45	18.69	19.05	19.28	19.42
Methyl 5,8-octadecadienoate	17.43	18.38	17.38	18.38	18.71	19.06	19.27	19.40
Methyl 6,9-octadecadienoate	17.46	18.44	17.40	18.46	18.81	19.19	19.37	19.57
Methyl 7,10-octadecadienoate	17.44	18.46	17.38	18.46	18.80	19.23	19.42	19.63
Methyl 8,11-octadecadienoate	17.48	18.53	17.42	18.51	18.87	19.28	19.47	19.65
Methyl 9,12-octadecadienoate	17.50	18.60	17.47	18.57	18.95	19.38	19.55	19.75
Methyl 10,13-octadecadienoate	17.60	18.70	17.56	18.60	19.03	19.46	19.69	19.81
Methyl 11,14-octadecadienoate	17.68	18.82	17.63	18.75	19.15	19.62	19.83	20.00
Methyl 12,15-octadecadienoate	17.78	18.90	17.72	18.88	19.28	19.75	19.97	20.16
Methyl 13,16-octadecadienoate	18.00	19.27	17.95	19.20	19.68	20.25	20.37	20.60
Methyl 14,17-octadecadienoate	17.80	19.04	17.75	18.95	19.33	19.78	19.96	20.18

[a] Capillary column.
[b] Packed column.
[c] Some sample decomposition also occurs.

distance of the double bonds from the carboxyl group, though there are sharp discontinuities for the 3,6- and 13,16- isomers where the ECLs are considerably higher than those of the adjacent isomers. A similar feature was noted with the methyl octadecenoates[8] where the ECLs of the 3- and 16- isomers were also much higher than those of nearby isomers. Such discrepancies are not seen in the ECLs of the isomeric methyl hydroxy and acetoxy palmitates[11], hydroxy, acetoxy and oxo-stearates[12] and methylene octadecanoates[13]. With the isomeric methyl-branched octadecanoates[14,15], similarly elevated ECL values are found for the 4- and 16-isomers. No explanation of this

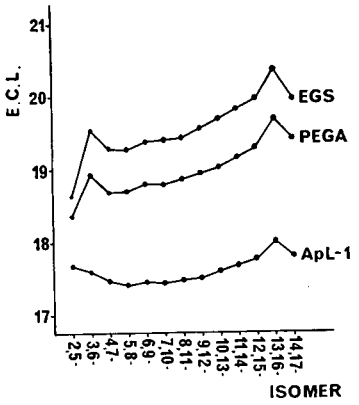

Fig. 1. Relationship between ECL for each isomer and double position on EGS, PEGA and ApL columns.

phenomenon can at present be offered. With the ApL columns, the ECL values conform to a gentle curve with a minimum at the 5,8-isomer, though the 13,16-isomer again has a greater ECL value than those of the isomers on either side. ECLs on the capillary ApL column are uniformly 0.05 higher than those on the packed column. The use of capillary columns is reported to result in losses of polyunsaturated fatty acids[16]. This was certainly so with methyl 2,5-octadecadienoate, which is the most labile of the isomers chemically. When this was chromatographed on the ApL capillary column, a number of late running spurious peaks were observed.

If the data of GUNSTONE, ISMAIL AND LIE KEN JIE[8] are used to calculate the increment in chain lengths for double bonds in each position (*i.e.* ECL—18) and thence, by adding these, to predict ECL values for the dienoic esters, values of the correct order of magnitude are obtained. However, in each instance, the calculated value was somewhat lower than that actually found. If the value for the 2,5-isomer is omitted, a mean difference of 0.13 is obtained for the ApL column, 0.16 for the NPGS column and 0.18 for the DEGS column. This may mean that there is some interaction between the double bonds which increases the dipole moment of the unsaturated system. Alternatively, the two double bonds may polarise the diallyl methylene group, again increasing the dipole moment of the whole and hence lengthening the retention time of the dienoic esters.

HOFSTETTER, SEN AND HOLMAN[7] have noted that for many unsaturated esters the difference between ECL_{EGS} and ECL_{ApL} is approximately constant per double bond in the molecule. This can only be so, however, if the graphs of double bond position against ECL of the isomers for the two liquid phases are parallel. As can be seen from Fig. 1, this is not so, but the relationship does have some validity when the double bonds are near the middle of the molecule. If such values are again calculated from the monoene data[8] and summed to predict similar values for the octadecadienoates, values are obtained that agree closely with those actually found, particularly with $ECL_{NPGS} - ECL_{ApL}$ where the agreement is within 0.05 for the 2,5- to 11,14-isomers. If this relationship can be shown to hold also for more unsaturated esters, it may be of value in the tentative identification of unknown fatty acids.

ECL values of the 8,11- to 12,15- isomers have been reported elsewhere[7] for ApL, DEGS and EGS columns. The ApL values agree within 0.06 and this probably reflects the particular stability of this liquid phase. Quite large discrepancies exist, however, between the values reported here and those of the previous report for polyester columns. This may be partly due to the fact that nitrogen was the carrier gas in the study now reported and helium was used by HOFSTETTER, SEN AND HOLMAN[7] or to differences in the ages or conditioning of the column packings. GUNSTONE *et al.*[8] similarly find good agreement between their values and those previously recorded by other workers for certain of the methyl octadecenoates on ApL columns. The use of common standards for correlations between laboratories is advised[8], however, and it would appear that ECLs on polar polyester phases may have less absolute value than has been supposed.

Both capillary columns gave excellent separations of isomers, representative examples of which are illustrated in Fig. 2. The ApL column (*ca.* 40,000 theoretical plates) separated isomers differing in ECL by 0.04 and the NPGS column (*ca.* 15,000 theoretical plates) those differing in ECL by about 0.08. LANDOWNE AND LIPSKY[17] have also described the separation of the methyl 8,11- to 11,14- octadecadienoates on polar and non-polar capillary columns. In theory, a capillary column could be con-

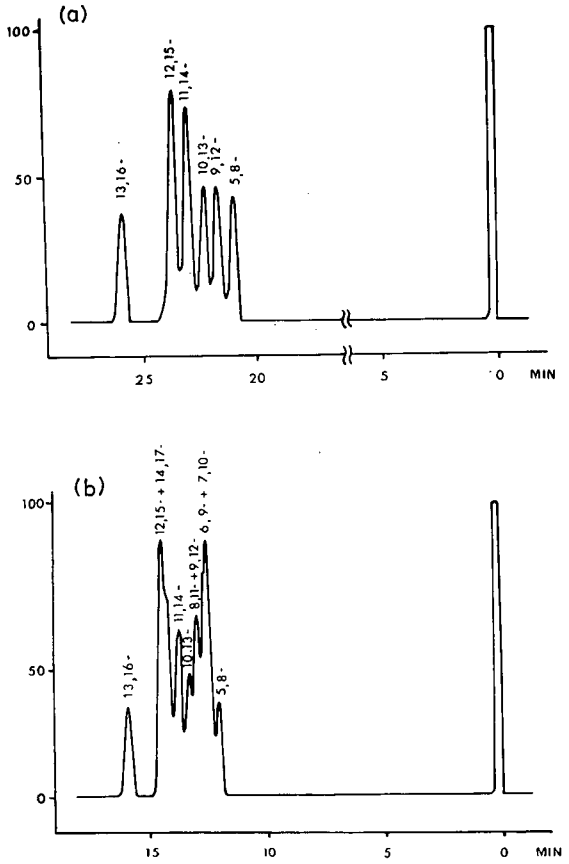

Fig. 2. GLC recorder tracing of mixtures of isomers on the capillary columns. (a) = ApL. (b) = NPGS.

structed from one or other of the liquid phases investigated to separate any two of the isomers with adjacent double bond systems; only the 4,7- to 7,10-isomers should cause any difficulty. For example, DEGS or EGS capillary columns should easily separate the 5,8-, 6,9-, 8,11- and 9,12- octadecadienoates which co-occur in the tissues of fat-deficient rats[18].

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CHROM. 3636

THE EVALUATION OF A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PLASMA TESTOSTERONE USING NICKEL-63 ELECTRON CAPTURE DETECTION

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SUMMARY

A method is described for the determination of testosterone in human peripheral venous plasma. The procedure involves addition of a labelled internal standard, mild saponification with sodium hydroxide, extraction with diethyl ether and preliminary purification on thin-layer chromatography. After formation of the heptafluorobutyrate derivative, the extract is rechromatographed on silica gel, followed by gas–liquid chromatography using a solid injection technique, Nickel-63 electron capture detection and electronic digital integration. The percentage error associated with each part of the procedure has been estimated and the total theoretical random error determined for each assay. In addition, the practical errors have been determined by replicate analyses. The method has been applied to the determination of testosterone in plasma from 41 healthy males (mean 528 $\pm$ 261 ng/100 ml plasma) and 20 healthy females (mean 40 $\pm$ 14 ng/100 ml plasma).

INTRODUCTION

Methods for the determination of testosterone (17β-hydroxyandrost-4-ene-3-one) in human peripheral venous blood have involved the use of fluorimetry, double isotope dilution, and gas–liquid chromatography with either flame ionisation or electron capture detection[1]. In general, the gas–liquid chromatographic methods are less time-consuming, and the selectivity and potential sensitivity of the electron capture detector appears most suitable for the accurate determination of testosterone in the systemic blood of healthy women. The present report is concerned with a theoretical and practical evaluation of a method involving the formation of testosterone heptafluorobutyrate followed by thin-layer chromatography, gas–liquid chromatography, and Nickel-63 electron capture detection.

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The high electron affinity of steroid heptafluorobutyrate was first reported by CLARK AND WOTIZ[2] in 1963, while NAKAGAWA *et al.*[3] investigated the properties of several halo-alkyl derivatives of testosterone and concluded that the heptafluorobutyrate was one of the most sensitive and stable. At the same time other reports by EXLEY[4] and VERMEULEN[5] outlined methods involving heptafluorobutyrate derivative formation for the determination of testosterone in small samples of human peripheral plasma, and VAN DER MOLEN *et al.*[6] have suggested modifying the method of BROWNIE *et al.*[7] by replacing formation of the monochloroacetate with the more sensitive heptafluorobutyrate.

A study of the formation of testosterone heptafluorobutyrate and the stability of this derivative during thin-layer chromatography, has been the subject of a preliminary report (WYMAN AND COLLINS)[8].

The operation and design of an electron capture detector was first reported in 1960[9], and subsequently modified to operate with a pulsed collection voltage[10]. However, the use of tritium foil as a β-emitter impeded its extended application to steroid analysis as a significant loss of tritium may occur at temperatures above 200°. The choice of Nickel-63 [11] as a radioactive source, and a modification of the detector arrangement[12] has enabled the use of temperatures up to 350°. This allows higher column temperatures to be employed, and the detector may readily be cleaned by raising the temperature over-night. The use of such a detector for the determination of steroid derivatives and an investigation into the operating conditions has previously been reported[8].

MATERIALS

Solvents and reagents

Diethyl ether (peroxide free) was redistilled immediately before use. Benzene, ethyl acetate, methanol, acetone, hexane and tetrahydrofuran (all analar grade) were redistilled and stored over granular anhydrous sodium sulphate.

Heptafluorobutyric anhydride was obtained from K and K Laboratories, Inc., Jamaica, N.Y.

Eastman chromagram sheets 6060 (silica gel with fluorescent indicator) were supplied by Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y.

The liquid phase for gas–liquid chromatography Xe-60 (cyanoethyl methyl silicone) and the support—Gas Chrom Q— were obtained from Applied Science Laboratories, Mc. State College, Pennsylvania.

Standards

Testosterone-4-^{14}C s.a. 55.2 mC/mmole and testosterone-7α-^{3}H s.a. 1.63 C/mmole were obtained from the Radiochemical Centre, Amersham, Bucks., Great Britain.

A sample of testosterone heptafluorobutyrate was kindly supplied by Dr. H. J. VAN DER MOLEN, University of Utrecht, The Netherlands. Subsequent batches of testosterone heptafluorobutyrate were prepared in the department by the method of VAN DER MOLEN *et al.*[6].

METHOD

A flow sheet of the method is shown in Fig. 1.

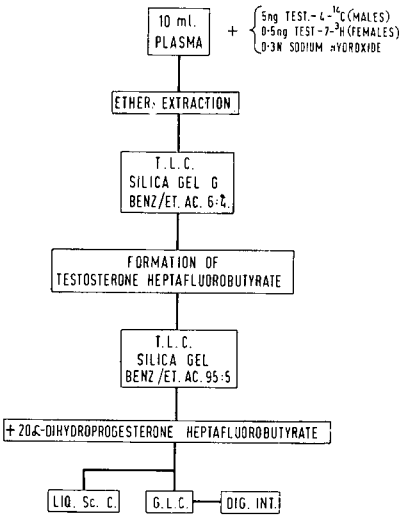

Fig. 1. Flow diagram of method.

Extraction

Peripheral venous blood (15–20 ml) is withdrawn by a non-greased syringe, transferred to a lithium heparin bottle, and centrifuged. The plasma is removed, and the volume measured. The sample may be processed immediately or stored at −15°.

An internal standard of 5 ng testosterone-4-^{14}C is added to plasma from male subjects, and 0.5 ng testosterone-7α-^{3}H to plasma from female subjects; an equal aliquot is taken for liquid scintillation counting. All standards are added from a 100 μl syringe with a Hamilton PB600 attachment (Hamilton Co. Inc., U.S.A.), which delivers 1/50 of the total syringe volume with each depression. After the addition of an equal volume of $^{1}/_{3}$ N sodium hydroxide, the mixture is gently shaken, and extracted with 2 × 100 ml diethyl ether. The ether is washed twice with 20 ml distilled water, and evaporated to dryness. The extract is then transferred with 3 × 1.0 ml methanol–chloroform (1:1, v/v) to a conical test tube, and taken to dryness under vacuum (using a Buchler rotatory evapo-mix).

Thin-layer chromatography

Glass plates 20 × 20 cm are coated with Silica Gel G, activated and stored in a desiccator until use. The extracts are transferred to the plate with a glass capillary using 3 × 0.05 ml methanol–chloroform (1:1, v/v). The first solvent system is benzene–ethyl acetate (6:4, v/v), the solvent front being allowed to run 15 cm. The plates are then removed and thoroughly dried. The testosterone-4-^{14}C in extracts from male plasma may be located by autoradiography (Kodirex non-screen X-ray film, overnight), and the testosterone-7α-^{3}H in female plasma by extrapolation from side and central standards.

The silica gel containing testosterone is loosened with a microspatula; aspirated on to a filter disc (1cm diam., No. 3 grade), and eluted with 3 × 0.05 ml ethanol under reduced pressure. The eluate is then evaporated under vacuum and thoroughly dried in a vacuum desiccator.

Derivative formation

A reaction mixture containing 1.0 ml dry hexane, 40 μl dry tetrahydrofuran and 20 μl heptafluorobutyric anhydride is prepared with each batch of samples, and 100 μl of the mixture added to each tube. The tubes are heated at 50° for 30 min, and the reagent subsequently removed under vacuum in a desiccator.

Thin-layer chromatography of derivatives

The extracts are transferred to an Eastman chromogram sheet with 3 × 0.5 ml dry acetone, and developed in benzene–ethyl acetate (95:5, v/v). Both solvents were dried over granular sodium sulphate before use. Testosterone heptafluorobutyrate is located by autoradiography or extrapolation from side and central standards. The silica gel containing the testosterone heptafluorobutyrate is aspirated onto a filter disc, and the extract eluted with 3 × 0.5 ml dry acetone, which is evaporated under vacuum in a desiccator.

Addition of second internal standard

100 μl from a standard solution of 20α-dihydroprogesterone heptafluorobutyrate is added to each tube so that $^1/_{20}$ of an extract of male plasma (5 μl) and $^1/_{10}$ of an extract of female plasma (10 μl) will contain 2 ng of the 20α-dihydroprogesterone heptafluorobutyrate for gas chromatography.

Gas–liquid chromatography

The gas–liquid chromatograph was a Pye 104 model 84 with a Nickel-63 electron

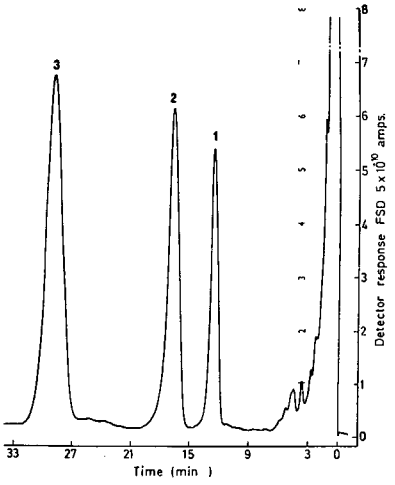

Fig. 2. Detector response following gas–liquid chromatography of 1.0 ng epitestosterone heptafluorobutyrate (peak 1), 1.0 ng testosterone heptafluorobutyrate (peak 2), and 2 ng 20α-dihydroprogesterone heptafluorobutyrate (peak 3).

capture detector. Glass columns 144 cm by 4 mm I.D. were packed with 3% Xe-60 on Gas Chrom Q, and operated at 215°. The carrier gas was 5% methane in argon at 50 ml/min. The detector was maintained at 225°, and every 150 μsec a 47 V pulse of 0.75 μsec duration was applied. These conditions gave a standing current of 1.7×10^{-9} A; the minimum level of testosterone heptafluorobutyrate detectable (a signal of twice background) was 0.005 ng, and the detector gave a linear response to 1.0 ng. The attenuation setting used for analysis produced a full scale deflection for 5×10^{-10} A. The samples are transferred to a solid injection syringe (SS60 $3\frac{1}{2}''$ needle, Hamilton Co. Inc., U.S.A.) and applied directly to the top of the column. The detector response was displayed on a chart recorder, and the peak heights and areas (by triangulation) were compared with the results from a digital integrator (Infotronics, Model CRS-10HB). The appropriate operating conditions for digital integration were selected for the typical response from an electron capture detector to steroid heptafluorobutyrates in biological samples. An automatic base-line drift corrector was used to overcome the difficulties arising from sloping base lines. Representative traces of standards and extracts are shown in Figs. 2, 3 and 4.

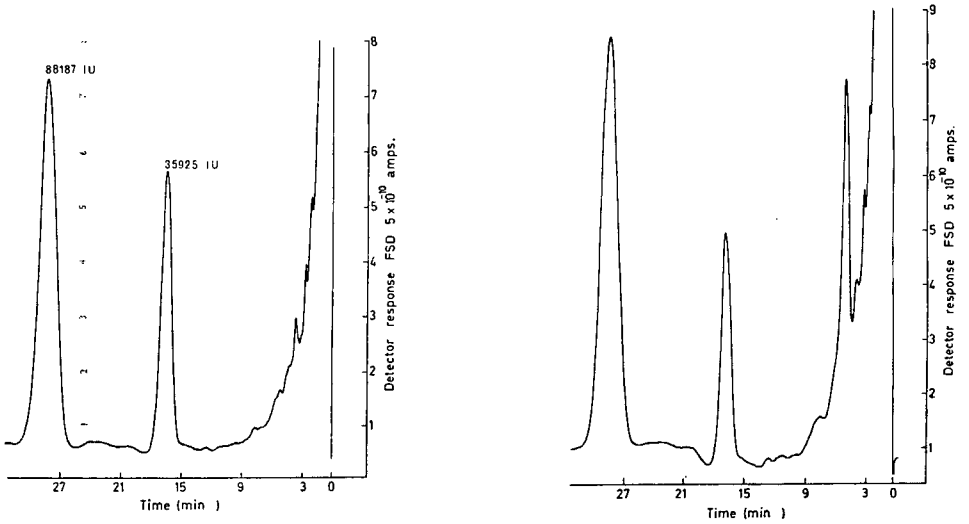

Fig. 3. Detector response following gas–liquid chromatography for 1/20th of the final extract from 10 ml of male plasma with the second internal standard.

Fig. 4. Detector response following gas–liquid chromatography for $^1/_{10}$th of the final extract from 10 ml of female plasma with the second internal standard.

Liquid scintillation counting

The remaining sample from each tube ($^{19}/_{20}$ from male plasma and $^9/_{10}$ from female plasma) is transferred to a potassium-free counting vial with 3 × 0.5 ml of methanol and dried. 8 ml of toluene containing 3.0 g/l of 2,5-diphenyl-oxazole (PPO) are added to the vials containing carbon-14 and 10 ml to the vials containing tritium. A liquid scintillation counting system—Nuclear Chicago Model 6860 (Mark 1)—was used, and each sample stabilised at 2° for 2 h, and a counting time selected to give a coefficient of variation of less than 1.5%. The counting efficiencies were determined

for each sample from calibration curves for an external standard channels ratio method using a ^{133}Ba source.

Calculation of results

A desk-top computer, the Olivetti Programma 101, was used to calculate the results according to the following formula:

$$S = \left[\left[\frac{C_s}{E_s} \cdot \frac{E_x}{\alpha C_x} \cdot \frac{I_s}{I_x} \cdot \frac{\beta T_x}{T_s} \cdot \frac{W_1}{W_2} \cdot A\right] - M\right] \cdot \frac{100}{V} \quad (1)$$

where:

C_s = counts/min of labelled standard,
E_s = counting efficiency for the labelled standard,
C_x = counts/min of the labelled standard in the extract,
E_x = counting efficiency for the labelled standard in the extract,
α = the aliquot taken for liquid scintillation counting,
I_s = detector response of the second internal standard,
I_x = detector response of the second internal standard in the extract,
β = the aliquot taken for gas–liquid chromatography,
T_x = detector response of unknown testosterone heptafluorobutyrate,
T_s = detector response of testosterone heptafluorobutyrate standard,
W_1 = molecular weight of testosterone,
W_2 = molecular weight of testosterone heptafluorobutyrate,
A = mass in ng of testosterone heptafluorobutyrate standard,
M = mass in ng of internal standard,
V = volume in ml of plasma,
S = ng of testosterone per 100 ml plasma.

Results

The method has been applied to the determination of testosterone in peripheral venous plasma taken from 20 healthy females (age 21–33 years) and from 41 healthy

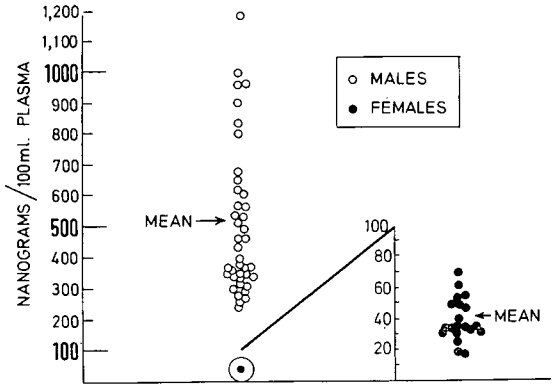

Fig. 5. Testosterone levels in peripheral venous plasma from 41 healthy males and 20 healthy females.

males (age 19–45 years). These values are shown in Fig. 5; the mean value for women was 40 ± 14 ng per 100 ml of plasma (range 18–71) and for men 528 ± 261 ng per 100 ml plasma (range 238–1195). In addition testosterone levels have been determined in plasma from women with idiopathic hirsutism, and in plasma from hypogonadal males before treatment. Some of these results have been used to assess the theoretical error involved in the determination of endogenous testosterone at values which lie between the normal female and male ranges (Fig. 6 and Table II).

Theoretical assessment of error in individual samples

In an attempt to determine the random error on individual samples the random errors on each term in equation (1) were assessed. The overall percentage error on S (e_S = (standard deviation × 100)/S) was then determined from equation (2) below, derived from the usual laws for the combination of additive and multiplicative independent normal errors.

$$e_S^2 = \left[\left[\frac{(e_{C_s}^2+e_{E_s}^2+e_{E_x}^2+e_{C_x}^2+e_s^2+e_{I_x}^2+e_{T_s}^2+e_{T_x}^2+e_A^2)(\Delta^2)+M^2 \cdot e_M^2}{\gamma^2}\right] + e_V^2\right] \quad (2)$$

where:

$$\Delta = \frac{C_s}{E_s} \cdot \frac{E_x}{\alpha C_x} \cdot \frac{I_s}{I_x} \cdot \frac{\beta T_x}{T_s} \cdot \frac{W_1}{W_2} \cdot A$$

$$\gamma = \Delta - M$$

and

e_{C_s} = the overall percentage error on C_s, and similarly for the other terms.

As α, β, W_1, and W_2 are constants, they do not not have any error, but contribute to the overall error in S because of the form of equation (2). The maximum error on the other terms in equation (1) were evaluated as follows:

C_s, C_x, E_s and E_x. There are two percentage errors in the term C_s, one from pipetting the standard into a counting vial, and the other from liquid scintillation counting. If the first aliquot from the PB600—100 µl syringe assembly is discarded, the subsequent pipetting error was found to be 1.25%, and as 20,000 counts were recorded for both carbon-14 and tritiated standards, the counting error was about 0.7%, assuming Poisson statistics.

These two errors were then combined to give:

$$e = (e_1^2 + e_2^2)^{\frac{1}{2}} = (1.25^2 + 0.7^2)^{\frac{1}{2}} = 1.4\%$$

There is a negligible pipetting error for C_x as the extract is washed into a counting vial. From 75 extracts a minimum of 3,000 counts and a maximum of 12,000 counts were recorded, which gave a range in percentage counting error of 0.9%–1.8%; the mean of 1.4% was used as the error in C_x for the calculation of total error in individual samples.

The error in the counting efficiency E_s and E_x is dependent upon the method used for evaluating the degree of quenching in a sample. This error was estimated to be 3% using an external standard channels ratio technique.

A, M, V. There are only three pipetting errors (all taken to be 1%) associated

with A, as the weighing error is negligible. This results in an overall error of 1.5%. The amount of labelled standard M added to the sample is determined from the liquid scintillation counting of an aliquot and the specific activity (in μC/mg) quoted by the Radiochemical Centre. As the error in the activity is 3% and that on the specific activity 1%, these combined with a 1% pipetting error give an overall error of 3.6%. The error on V was taken as a maximum of 5%.

I_s, T_s, I_x, T_x. I_s and T_s each have a 1% pipetting error in addition to the error in measuring detector response, while for I_x and T_x it is more complex, as there is a 1% pipetting error involved in adding the internal standard to the extract and a 1% pipetting error involved in removing both simultaneously; T_x is considered to have no other error besides that of the detector response, and I_x is considered to have two additional 1% pipetting errors.

The inherent error in the measurement of detector response has been evaluated using the three methods below.

(a) *Peak height.* Several factors contributing to the overall error in peak height have been considered. These included the base line variation, the selection of the base line position under the peak, the limitations in using a ruler, and the finite thickness of the trace. The resulting percentage error was calculated for the range of peak heights shown in Table I.

(b) *Peak area.* The error in peak area is compiled from that in peak height and from that involved in the determination of the peak width at half height. The error in peak width includes similar measurements as discussed above, and it is also influenced by the peak height. This is particularly important for small peaks where the uncertainty in the peak height is large and could lead to the measurement of the peak width at an incorrect position.

Under our conditions, the peak width for testosterone heptafluorobutyrate was about 4 mm, and the average error in the width was estimated to be 10% for all peak heights. The 20α-dihydroprogesterone heptafluorobutyrate gave a peak width of approximately 6 mm, and the average error in width ranged from 10% at a height of 10–30 mm to 6% for peak heights of 100 mm and greater.

These errors were then combined to give the overall error in area:

$$e^2_{area} = (e^2_{height} + e^2_{width})^{\frac{1}{2}}$$

and the values are shown in Table I.

(c) *Digital integration.* After attenuation the detector signal is amplified by the

TABLE I

PERCENTAGE ERRORS IN HEIGHT, WIDTH AND AREAS FOR TESTOSTERONE HEPTAFLUOROBUTYRATE AND 20α-DIHYDROPROGESTERONE HEPTAFLUOROBUTYRATE

Peak height (mm)	10	20	30	40	50	60	70	80	90	100	150	200
% Error (heights)	21.2	10.6	7.1	5.3	4.2	3.6	3.0	2.7	2.4	2.1	1.4	1.1
% Error (width) $w = 4$ mm	10	10	10	10	10	10	10	10	10	10	10	10
% Error (width) $w = 6$ mm	10	10	10	9	9	8	8	7	7	6	6	6
% Area error $w = 4$ mm	23.4	14.6	12.3	11.3	10.8	10.6	10.4	10.4	10.3	10.2	10.1	10.1
% Area error $w = 6$ mm	23.4	14.6	12.3	10.4	9.9	8.7	8.5	7.5	7.4	6.4	6.2	6.1

integrator and converted to pulses which are counted by a decade scaler. The count-rate corresponding to a full scale deflection of 1 mV is 60,000 counts/min. The integrator recognises the start of a peak from a combination of three factors—the rate, size, and duration of the signal increase. The electronic error in the integration of an ideal peak is quoted as 0.5%, but for biological extracts at high sensitivity the overall error is probably much greater. As it is difficult to assess the error in the integration units representing a single peak, the mean percentage error of 5.5% obtained from the integration of replicate standard samples at different levels (0.1 → 0.75 ng) was taken for all peaks.

These three methods of evaluating detector response have been used to determine the overall percentage error in the testosterone levels from 200 plasma samples; representative values are shown in Table II, and there is good agreement between the

TABLE II

VARIATION OF THEORETICAL PERCENTAGE ERROR WITH CONCENTRATION OF TESTOSTERONE FOR THREE METHODS OF DETERMINING DETECTOR RESPONSE

*Sample**	*ng/100 ml plasma*					
	Area	*% Error*	*Height*	*% Error*	*Integral units*	*% Error*
F	19.7 ± 5.0	23.3	21.8 ± 2.4	11.2	25.6 ± 4.1	16.2
F	33.4 ± 7.2	21.5	33.6 ± 3.1	9.2	31.7 ± 4.8	15.2
F	36.8 ± 7.8	21.1	36.6 ± 3.2	8.9	40.5 ± 5.9	14.6
F	36.9 ± 8.0	21.6	40.9 ± 3.7	9.2	42.4 ± 6.9	14.6
HF	107.7 ± 21.2	19.7	115.4 ± 10.1	8.7	101.0 ± 13.9	13.8
M	285.1 ± 75.2	26.4	294.9 ± 39.4	13.4	247.6 ± 41.6	16.8
M	312.1 ± 76.3	24.5	333.6 ± 40.0	10.8	434.6 ± 67.6	15.6
M	483.0 ± 109.0	22.6	498.1 ± 48.6	9.8	502.0 ± 77.4	15.4
M	693.0 ± 130.7	18.8	643.4 ± 53.5	8.3	657.1 ± 87.7	13.4
M	873.7 ± 175.9	20.1	931.7 ± 79.4	8.5	949.4 ± 133.8	14.1

* F = female, HF = hirsute female, M = male.

testosterone levels found by the three methods. The overall percentage error appears to be lowest for the peak height method; however, this is not the optimum way of calculating the concentration in this method, as a constant peak width is assumed for each biological sample, and this has not been found in practice.

In Fig. 6, the variation of overall percentage error is plotted against the concentration in ng/100 ml for the two levels of labelled standard (5 ng and 0.5 ng) added respectively. The percentage errors for the testosterone values are hypothetical below 18 ng/100 ml plasma, and were determined from representative values for recovery, standards, and peak areas for testosterone heptafluorobutyrate.

The shape of these curves are an inherent feature of equation (2), and result in errors that are virtually independent over the entire male range of testosterone levels and the majority of the female range, only increasing rapidly at the lower limit of each range. Those points lying well above the peak area curve in Fig. 6b are the result of abnormally low recoveries of the labelled standard, which led to a greater error in the measurement of the sample detector response.

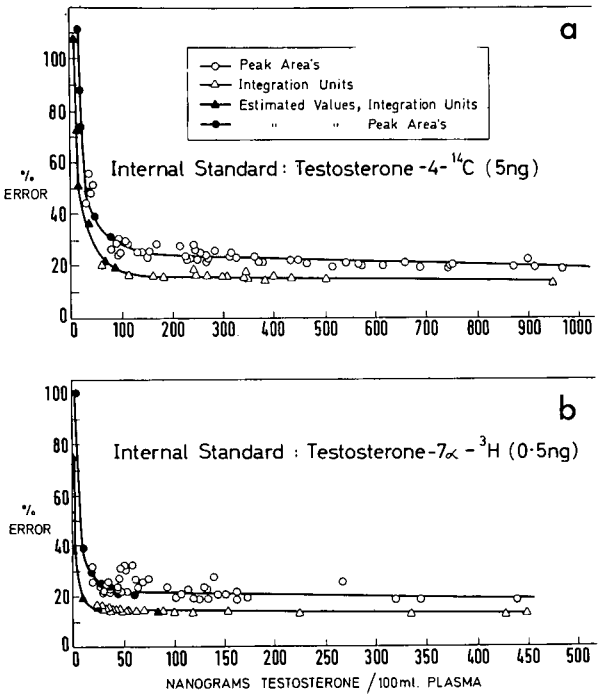

Fig. 6. The variation in theoretical random percentage error with plasma testosterone concentration.

Practical errors

After determining the theoretical error on individual samples, the practical errors were determined from recovery of known amounts of testosterone from water, and from replicate analyses on pools of male and female plasma.

Accuracy

The accuracy of the method was assessed by replicate recoveries of authentic testosterone added to 10 ml of distilled water. The results were calculated from peak areas, and expressed as ng/100 ml of water (Table III). The mean recovery from ten determinations at the 50 ng level was 94.8 ± 5.4%, while at the 4 ng level ten determinations gave a mean of 89 ± 32%.

Precision

The precision of the method was assessed from serial determinations on plasma pools taken from a 35 year old male blood donor and a 22 year old female donor. The detector response was measured by peak areas, and the results expressed as ng per 100 ml plasma. The mean value from the male donor was 258 ± 30 ng/100 ml plasma with a coefficient of variation of 11.6%, and the mean value from the female donor was 34.6 ± 5.45 ng with a coefficient of variation of 15.7% (Table III).

TABLE III

REPLICATE RECOVERY EXPERIMENTS AND ANALYSIS OF POOLED PLASMA

	Water, blank, ^{14}C STD.	*Water, recovery, 50 ng*	*Male, plasma, pool*	*Water, blank, ^{3}H STD.*	*Water, recovery, 4 ng*	*Female, plasma, pool*
	−20	488	310	7.1	50	32
	−21	456	250	18.4	27	29
	−10	494	252	9.1	42	45
	−19	481	223	8.6	30	37
	+16	525	247	8.6	28	43
	−28	454	303	10.6	25	31
	+ 9	484	268	5.6	25	31
	−37	472	240	8.6	27	31
	− 3	425	233	15.6	40	36
	−23	456			62	31
Mean ± S.D.	−13 ± 17	474 ± 27	258 ± 30	10.2 ± 4.1	35.6 ± 12.6	34.6 ± 5.45
Coefficient of variation		5.8	11.6		35.4	15.7

Sensitivity

The sensitivity of the method has been evaluated by relating the endogenous level of testosterone with the theoretical percentage error associated with its measurement. As can be seen from Fig. 6, if a 50% error is taken as that which defines the lower limit of sensitivity, then the lower limits (using peak areas) are 30 ng/100 ml plasma, when 5 ng of testosterone-4-^{14}C is taken as labelled internal standard, or 7.5 ng/100 ml plasma when 0.5 ng of testosterone-7α-^{3}H is taken. If a digital integrator is used these values may be reduced to 15 ng/100 ml plasma, and 2.5 ng/100 ml plasma.

TABLE IV

THE CHROMATOGRAPHIC MOBILITIES OF STEROIDS WITH SIMILAR PROPERTIES TO TESTOSTERONE

Steroid	*Thin-layer chromatography*		*Gas–liquid chromatography, 3% Xe-60*
	Rt (Benzene–ethyl acetate, 6:4)*	*Rth** (After heptafluorobutyrate formation) (Benzene–ethyl acetate, 95:5)*	*R_{ph}*** (After heptafluorobutyrate formation)*
19-Nortestosterone	0.82	0.95	0.53
Epitestosterone	0.91	0.87	0.45
Testosterone	1.00	1.00	0.60
20α-Dihydroprogesterone	1.09	1.06	1.00
Aetiocholanolone	1.09	1.72	0.25
17α-Hydroxyprogesterone	1.18	—	—
20β-Dihydroprogesterone	1.20	0.99	0.82
Dehydroepiandrosterone	1.36	1.68	0.28
Androsterone	1.43	1.67	0.22

* Rt = mobility relative to testosterone.
** Rth = mobility relative to testosterone heptafluorobutyrate.
*** R_{ph} = retention time relative to 20α-dihydroprogesterone heptafluorobutyrate.

Specificity

At the present time, the specificity can only be inferred, as it is difficult to assess the purity of each sample. The good separation of testosterone and its derivative from steroids possessing similar chromatographic properties is shown in Table IV. In addition, the low values for the water blanks (Table III) suggest that solvents and reagents do not interfere with the assay. Further evidence for specificity may be deduced from the fact that the range of values in healthy men and women are similar to those reported using other techniques[1].

DISCUSSION

The plasma testosterone levels from 41 healthy males were in the range 238–1195 ng/100 ml plasma, which is similar to that reported by other workers[1]. However, as 50% of the values were below 400 ng/100 ml plasma, the mean value of 528 ± 261 ng/100 ml plasma is slightly lower than that obtained by other methods. The plasma testosterone levels from 20 healthy females were within a narrow range, 18–71 ng per 100 ml plasma, with a mean value of 40 ± 14 ng/100 ml plasma. This value and range is similar to those reported by VAN DER MOLEN *et al.*[6] using electron capture detection, and by LOBOTSKY *et al.* (1964)[13] using the lengthy double isotope dilution derivative method of RIONDEL *et al.*[14].

The method is similar in principle to that described by BROWNIE *et al.* in 1964[7]; however, the use of the more sensitive heptafluorobutyrate derivative and the thermally stable Nickel-63 detector followed by digital integration of the response has led to more precise measurements on testosterone levels in female plasma. Furthermore, this method is less time-consuming than those based upon the principle of double isotope dilution, and can be performed with similar accuracy and precision. Recovery experiments (4 and 50 ng) suggest that the present technique slightly underestimates the testosterone level, but both recoveries are within the standard deviation of the expected value. The precision of nine determinations upon a pool of male plasma was 11.6%, and 15.7% for ten determinations upon a pool of female plasma. From Fig. 6 the theoretical errors for the corresponding testosterone levels are 24.5% and 24.0%; this difference in practical and calculated error is reasonable, as all the samples from the pool were assayed simultaneously under optimum conditions which would result in a smaller practical random error.

Another advantage of this method and the use of equation (1) to calculate the results, is that the theoretical error (and, by inference, the practical error) is essentially constant over the testosterone levels of interest. All the terms in equation (2) influence the magnitude of the error for a particular testosterone level; this is well illustrated by the difference in the errors for peak areas and integrator units. In all cases, the use of an integrator, which implies smaller errors on the terms concerning detector response, has led to a significant decrease in the overall error. In addition to this general effect, one factor in equation (1)—the quantity of labelled internal standard added—greatly influences the overall error, as in equation (2) the magnitude of this term appears as well as the error associated with it. This effect is illustrated in Figs. 6a and 6b. In Fig. 6a, 5 ng of testosterone-4-^{14}C was added as the labelled standard, and the theoretical error begins to increase rapidly for testosterone levels under 75 ng/100 ml. However, with 0.5 ng of testosterone-7α-^{3}H as labelled standard, the error begins to increase rapidly

for testosterone levels of less than 25 ng/100 ml plasma. Thus, by reducing tenfold the amount of labelled standard added, it is possible to achieve results in the normal female range with the same accuracy as is obtainable for values in the normal male range. As it is difficult to locate tritium accurately on thin-layer chromatograms, the method would be improved by the availibility of testosterone-4-^{14}C of much higher specific activity.

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CHROM. 3643

ANALYSIS OF ANTIBIOTICS BY GAS CHROMATOGRAPHY

I. LINCOMYCIN

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SUMMARY

The analysis of lincomycin and lincomycin B by an improved gas–liquid chromatographic method is described. The trimethylsilyl ether is formed and extracted before chromatography. The method has been applied successfully to bulk material and has been extended to determine the lincomycin content of capsules, syrups, injectables, and powders. The results compare favorably with those obtained by microbiological assay.

INTRODUCTION

Lincomycin is an antibiotic, produced by *Streptomyces lincolnensis* fermentation, which inhibits the growth primarily of Gram-positive bacteria. Its discovery and biological properties were reported by MASON *et al.*[1] and its chemical structure was elucidated by HERR AND BERGY[2] and HOEKSEMA *et al.*[3]. A congener, lincomycin B*, is produced concomitantly with lincomycin at a level of about 3% during biosynthesis[4]. The only structural difference between the two analogs is that lincomycin has a propyl substituent at position 4 on the pyrrolidine ring and lincomycin B has an ethyl group[5,6].

Lincomycin may be assayed microbiologically, either directly[7] or after isolation by thin-layer chromatography[8]. The official method used by the Food and Drug Administration in the certification of lincomycin hydrochloride monohydrate bulk and dosage forms is the microbiological assay[9]. An automated chemical assay reported recently[10] is based upon acid cleavage of the antibiotic, followed by determination of the liberated methanethiol with a disulfide color reagent. However, these methods lack specificity and lincomycin cannot be differentiated from lincomycin B or from a number of other antibiotics. Lincomycin B possesses the same antibacterial spectrum as lincomycin, but has only about 25% as much activity[11].

* Also known by the code number U-21699.

HOUTMAN *et al.*[4] developed a gas–liquid chromatographic method for the determination of both lincomycin and lincomycin B. The method employs silylation in pyridine and direct chromatographic analysis. Many determinations were performed in this laboratory using their method but several major difficulties were encountered.

One difficulty was that, since the silylating reagent reacts with practically all compounds with labile protons, lincomycin must be separated from significant amounts of substances containing groups such as hydroxyls, primary or secondary amines, and sulfhydryls. These compounds occur in dosage forms as solvents (water, glycols), saccharides (sucrose, lactose), and various additives such as flavors and preservatives. Individual adjustments were described[4] to compensate for extensive recognized interferences; for example, freeze-drying was used to eliminate the interference from water. However, this procedure does not remove sugars.

Because of the excessive silylating reagent, the electrode in the detector system of the gas chromatograph rapidly became contaminated, and the efficiency of the entire analysis was reduced considerably. The coating effect, also previously reported by other workers[12–16], could not be eliminated by injecting Freon 12 into the column as recommended by the manufacturer. After every four to five sample injections, the electrode had to be removed and cleaned to avoid gross impairment of electrode sensitivity and distortion of instrument response. This frequent cleaning resulted in undue damage to the electrode because of excessive handling, in the necessity of restandardization after each cleaning operation, and in premature replacement of the electrode.

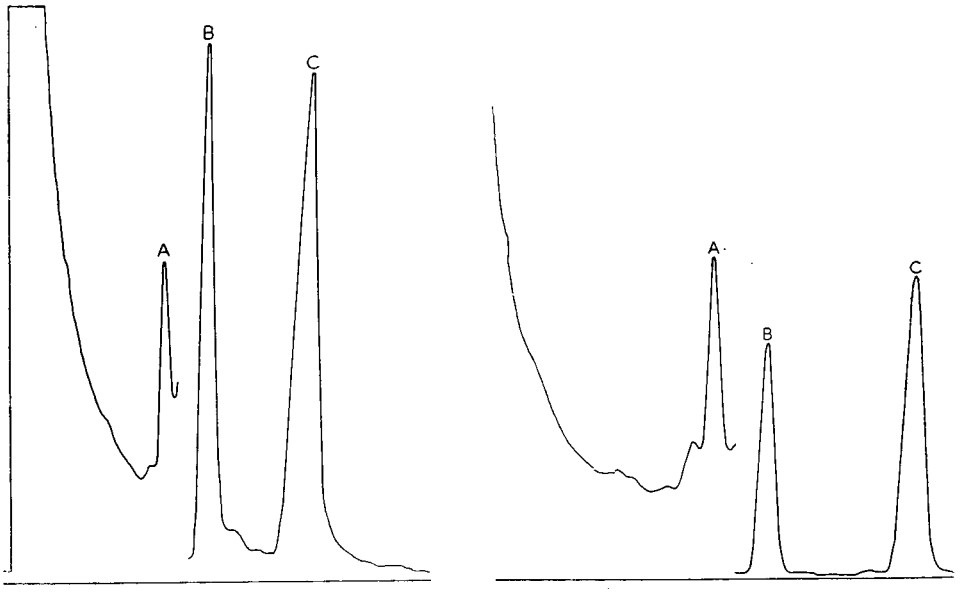

Fig. 1. Direct injection of silylated compounds in pyridine solvent. Glass column, 4 ft., 3 mm I.D., packed with 3% SE-30 on Diatoport S; column temperature, 240°; carrier, nitrogen at 51 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Fig. 2. Direct injection of silylated compounds in pyridine solvent. Glass column, 6 ft., 3 mm I.D., packed with 9.1% DC-200 on 80/100 mesh Gas Chrom Q: column temperature, 262°; carrier, nitrogen at 30 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Another difficulty was that the pyridine used as a solvent during preparation of the silyl derivative caused serious tailing in the chromatograms in the direct injection method (Figs. 1 and 2).

The method was evaluated in an attempt to minimize these difficulties, and was modified by extracting the silyl ether derivative into cyclohexane. A new method of preparing samples of dosage forms was developed and the applicability of the modified methods was studied.

EXPERIMENTAL

Apparatus

Gas chromatograph. A Barber-Colman Model 5000 gas chromatograph with a flame ionization detector was used; recorder, 5 mV range and $^1/_3$ in./min chart speed. Glass column: U-shaped, 6 ft. by 3 mm I.D., packed with 5% SE-30 on Gas-Chrom Q (80/100 mesh). Operating conditions: column temperature, 257°; detector temperature, 280°; injector temperature, 280°; carrier gas, nitrogen at 20 p.s.i., 150 ml/min; pressure of hydrogen, 32 p.s.i.; air, 40 p.s.i. (adjusted for maximum response). Current 2×10^{-8} A F.S.D.; sensitivity, 100, and attenuation, 2.

Reagents

Solvents. Anhydrous methanol, absolute ethanol, and cyclohexane, all reagent grade. Pyridine, also reagent grade, kept over potassium hydroxide.

Silylating reagent. Nine parts of hexamethyldisilazane mixed with one part of trimethylchlorosilane. The mixture is cleared by filtration.

Lincomycin standard. About 4 mg of lincomycin reference standard, accurately weighed and transferred to a centrifuge tube.

Internal standard. A saturated solution of tetraphenylcyclopentadienone* prepared in cyclohexane, and cleared by filtration.

Sample preparation

Bulk materials. Samples are prepared in the same manner as the lincomycin standard above.

Injections and sensitivity powders. The samples are diluted with or dissolved in methanol. An aliquot containing approximately 4 mg lincomycin is transferred to a centrifuge tube and is evaporated to dryness on a steam bath under a current of dry air.

Capsules. The empty capsule and its contents are added to methanol. The lincomycin is dissolved with gentle heat; the mixture is cooled and further diluted with methanol. After the sediment has settled, an aliquot of the supernatant containing about 4 mg of lincomycin is transferred to a centrifuge tube and evaporated to dryness as above.

Syrups. An aliquot of 5 ml of syrup is diluted at least 20-fold with absolute ethanol. The solution is heated, then cooled overnight to precipitate the sucrose. A portion of the supernatant is further diluted with methanol. An aliquot of the final dilution containing about 4 mg of lincomycin is transferred to a centrifuge tube and evaporated to dryness as above.

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Derivatization

A lincomycin standard is treated in the same manner as the samples. Each dry or dried sample is dissolved in 1 ml of pyridine, and 0.2 ml of the silylating reagent is added. The reaction mixtures are allowed to stand not less than 30 min, 1.00 ml of the internal standard solution and 2 ml of water are added, and the mixture is shaken vigorously. The phases are separated by gravity or centrifugation.

Chromatography and calculations

Five microliters of the cyclohexane phase are injected into the gas chromatograph. The areas of each peak are measured by planimetry or by disc integration.

The lincomycin content is determined by direct comparison of the ratio of the peak areas (lincomycin:internal standard) with that of the lincomycin reference standard treated in an identical manner. Lincomycin B content in bulk material is determined as a fraction of the combined lincomycin + lincomycin B.

RESULTS AND DISCUSSION

The retention times of lincomycin, lincomycin B, the internal standards, and the residual sugars are shown in Table I. Under specified experimental conditions, the retention time of a particular entity is a good qualitative characteristic. Occasionally, identification of a substance may become complicated by the appearance of more than one major peak due to the formation of multiple derivatives, such as occurs with salicylic acid[17,18] or by the presence of such anomers as α- or β-lactose[19]. Various parabens[12,20], carbohydrates[19], and the possible interference of water[21] have been studied by silylation and gas chromatography. Under the conditions of this study, no qualitative interference has been found from any substance presently used in compounding lincomycin preparations.

TABLE I

RETENTION TIMES OF LINCOMYCIN AND OTHER COMPOUNDS

	Retention time (min)	*Relative retention time*
Lincomycin	7.65	1.00
Lincomycin B	6.12	0.78
Lactose	4.11, 6.00	0.54, 0.78
Sucrose	4.35	0.57
Internal standard	11.10	1.45

The improved method was used for single determinations of lincomycin samples, and the results were compared with the average of several microbiological assays (Tables II and III). The peak areas were obtained by a disc integrator and were normalized by the internal standard technique. The results of analyses of bulk samples of the various dosage forms show good correlation between the chromatographic and microbiological assays.

By using methanol as a solvent, most of the lactose present can easily be separated

TABLE II

ANALYSIS OF LINCOMYCIN HYDROCHLORIDE MONOHYDRATE BY IMPROVED METHOD

Sample No.	*GLC*		*Microbiological assay (μg lincomycin/mg)*	
	μg lincomycin/mg	*Lincomycin B (%)*	*Lab 1*	*Lab 2*
1	847	3.7	882	887
2	847	3.5	898	905
3	828	4.7	908	888
4	903	2.6	897	874
5	843	3.0	886	883
6	824	4.0	829	874
7	868	4.7	892	874
8	868	4.5	871	874
9	862	2.2	880	888
10	856	2.4	881	873
11	846	1.8	886	870
12	849	1.5	911	876
13	849	3.5	885	853
14	834	2.0	902	866
15	817	3.3	904	867
16	846	2.8	873	868
17	846	3.7	890	859
18	845	3.4	872	860
19	871	4.2	860	853
20	839	3.7	873	854
21	869	3.4	889	854
22	862	4.4	898	856
23	865	4.0	900	862
24	849	3.8	899	851
25	845	3.8	906	867
26	843	4.4	858	863
27	854	3.1	906	864
28	858	2.7	880	861
29	862	3.4	887	856
30	840	4.3	864	867
31	851	4.1	865	874
32	859	4.4	848	852

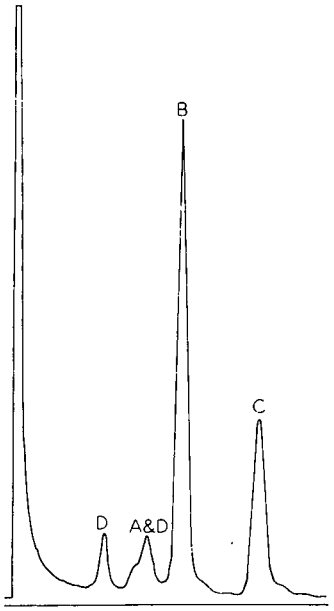
from lincomycin. As shown in Fig. 3, small amounts of residual lactose do not interfere with the gas chromatographic resolution of lincomycin.

Although sucrose in lincomycin syrup causes interference, diluting the syrup with absolute ethanol precipitates most of the sucrose, in time. Precipitation is improved and accelerated by subjecting the alcoholic solution to ultrasonic vibrations. Separation of the lincomycin from most of the sucrose can be achieved also by extracting an alkaline solution of the sample with chloroform (Fig. 4).

Water in lincomycin hydrochloride injection reacts with the silylating reagent to produce primarily trimethylsilanol. Water was eliminated simply by diluting the sample with methanol and evaporating an aliquot to dryness.

Small amounts of residual water, sugars, and other additives do not interfere, if sufficient silylating reagent remains to react quantitatively with the antibiotic (see Fig. 5). Fig. 6 shows that insufficient separation from sucrose yielded a recovery of lincomycin as low as 73%.

The silylation of compounds with labile protons is a reaction which is useful in



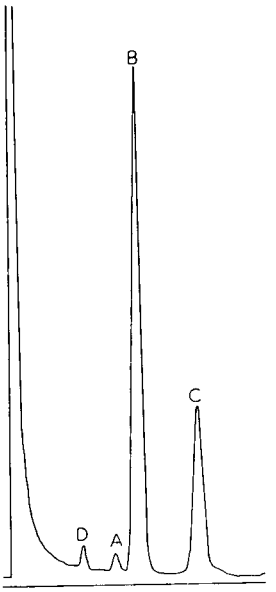
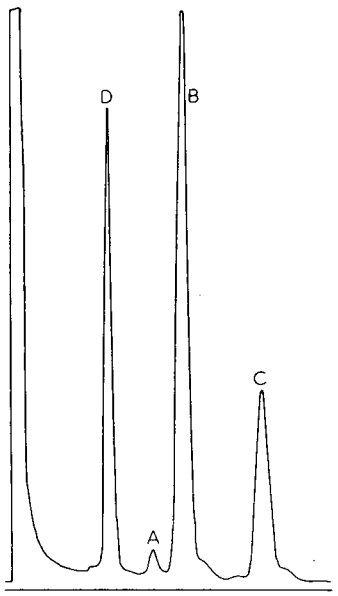


Fig. 3. Chromatogram of silylated compounds from capsules by the improved method. Key: A — lincomycin B; B = lincomycin; C — internal standard; D = lactose.

Fig. 4. Chromatogram of syrup sample extracted with chloroform. Column, 5% SE-30 on Gas Chrom Q; column temperature, 255°; carrier, nitrogen at 150 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard; D = sucrose.



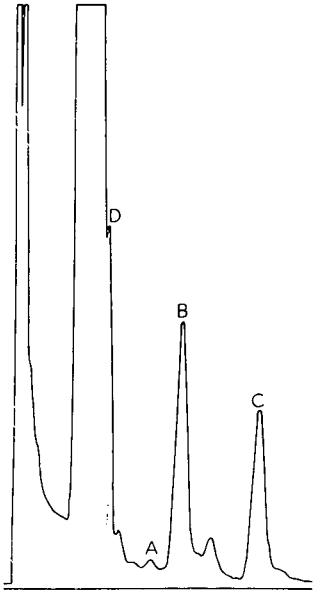


Fig. 5. Syrup with most of the sucrose removed before silylation; improved method, conditions as in Fig. 3. Key: A = lincomycin B; B — lincomycin; C = internal standard; D = sucrose.

Fig. 6. Syrup with excess sucrose in reaction medium showing incomplete recovery of lincomycin by the improved method. Key: A — lincomycin B; B = lincomycin; C = internal standard; D = sucrose.

TABLE III

ANALYSIS OF LINCOMYCIN IN DOSAGE FORMS BY IMPROVED METHOD

Sample No.	*GLC (mg)*	*Microbiological assay (mg)*	
		Lab. 1	*Lab 2*
	Syrup (5 ml)		
1	234	250	252
2	268	238	243
3	255	238	243
4	254	250	245
5	271	251	245
	Injection, syringe		
1	642	629	
2	627	629	
3	699	632	
4	694	632	
5	655	622	
6	655	630	
	Injection, vial (ml)		
1	323	304	327
2	328	308	319
3	330	306	320
4	307	313	314
5	299	311	330
6	334	316	323
7	323	306	341
	Capsule		
1	250	250	251
2	514	515	507
3	498	496	496
4	505	502	488
5	513	505	513
6	265	245	250
7	265	254	251
8	538	505	500
	Powder, vial		
1	20.7	21.4	20.3

organic analysis, and its use in gas chromatography, thin-layer chromatography, and infrared and mass spectroscopy is well established[22]. Silylation of organic compounds tends to increase volatility, thermal stability, and solubility in aprotic solvents. The original compound is easily converted to and recovered from the trimethylsilyl ether derivative[23,24].

Various solvents for the silylation reaction were studied. Recoveries indicated that silylation of lincomycin was only about 10% complete in dimethyl sulfoxide and from 75 to 100% complete in ethyl acetate, acetone, and acetonitrile. Based on overall qualitative and quantitative performance, acetonitrile was comparable to pyridine. Pyridine is preferred over other solvents because of its solubilizing power.

A mixed silylating reagent was employed for convenience. CARTER AND GAVER[25] have previously shown that using a silylating reagent mixture for long-chain bases

resulted in more reproducible and reliable analyses than when the reagents were added individually.

The silyl ether derivative of lincomycin was extracted into cyclohexane in an attempt to seperate it from any possible interfering substances present in antibiotic preparations. This extraction vastly reduces solvent (pyridine) tailing and chromatographic peaks are symmetrical. The water added during the extraction provided an immiscible phase which scavenges excess silylating reagent, resulting in a cleaner electrode, and dissolves the ammonium chloride formed during the silylation, eliminating the occasional sticking of solids in the microsyringe.

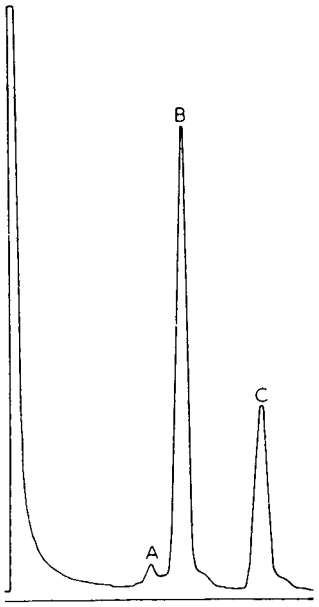


Fig. 7. Chromatogram of lincomycin derivative by the improved method; conditions as in Fig. 3. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Two stationary phases were investigated. Although DC-200 (Fig. 2) was superior to SE-30 (Fig. 1), the latter was used for routine work because of its reportedly greater thermal stability. Other combinations of inert phases and supports were not studied.

A saturated solution may not be the best choice for an internal standard, but it is dictated in this case because of the relatively low solubility of tetraphenylcyclopentadienone in cyclohexane. When the samples and standard are prepared at the same time, the actual concentration of the internal standard is inconsequential, as long as the peak areas can be normalized. This procedure has worked well in practice.

During the initial phase of this study, seven bulk lots and two samples of lincomycin injection were analyzed by the direct injection technique[4]. Five methods of quantitation were used with and without the internal standard.

All these calculation methods give comparable values and the injection volume seemed to be replicated well enough to indicate that the internal standard may not always be necessary. However, in routine work it is recommended that an internal

standard be used as a built-in check of the instrument, the operator, and the procedure. The use of an integrator, mechanical or electronic, is desirable.

Initially, because changes in the attenuation of the recorder precluded the use of the disc integrator in our system, the lincomycin B content was calculated as a fraction of the total area by planimetry (Figs. 1 and 2). The response factors of each lincomycin were assumed to be essentially equal because of the close similarity of the structures and the amounts of carbon in the molecules. In the improved method, the attenuation remains fixed and an accurate disc integrator value can be obtained (Fig. 7).

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CHROM. 3658

CHROMATOGRAPHY OF SOME CYSTINE PEPTIDES AND FORMATION OF MIXED DISULFIDES

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SUMMARY

Chromatographic systems for cystine peptides were established and mixed disulfide formation was studied using a Technicon Amino Acid Analyzer. The ninhydrin molar color yield of peptides with N-terminal cystine was increased by splitting the disulfide bond with sodium bisulfite.

Cystine peptides were prepared in the sulfhydryl form by reducing the disulfide bonds with dithiothreitol. The reduced peptides were separated from dithiothreitol on a small column of Dowex 50W X4 in the H^+ form. The sulfhydryl peptides were mixed together and allowed to oxidize completely. The oxidized products were analyzed in the chromatographic system. The formation of symmetrical and mixed disulfides from the sulfhydryl compounds appeared to occur randomly.

INTRODUCTION

The biological significance of disulfide bonds and sulfhydryl groups has two main aspects. Disulfide bonds play an important role in maintaining the conformation of proteins[1]. Sulfhydryl-containing compounds have been shown to provide protection for a variety of organisms against radiation-induced damage[2]. While studying the mechanism of the protective properties of sulfhydryl compounds, ELDJARN AND PIHL[3,4] showed the formation of some mixed disulfides. Recently, VAN RENSBURG AND SWANEPOEL[5] showed that when bisulfite (HSO_3^-) ruptures mixed disulfides, steric factors sometimes cause the SO_3^{2-} group to attach preferentially to one of the two resulting thiol compounds. Further study of mixed disulfides has been limited by the techniques available. This paper reports the use of ion-exchange chromatography to study disulfide formation from cysteine-containing peptides.

The work consisted of three main problems. First, a chromatographic system for analyzing the amounts of peptides had to be established. Because the commercially

* From a thesis submitted to the Graduate Faculty of Wake Forest University in partial fulfillment of the requirements for the degree of Master of Science.

available compounds were symmetrical disulfides, the second problem was to devise a method of reducing the disulfides to isolate the peptides in their sulfhydryl form. The third phase of the work was the actual formation of mixed disulfides from the reduced peptides and the chromatographic analysis of the products. Because the mixed disulfides formed were to consist of two peptides of differing amino acid composition, we were curious to see if steric factors would favor or hinder any particular disulfides from forming.

MATERIALS AND METHODS

Cystine-containing peptides were obtained from Cyclo Chemical Corp. (Los Angeles, Calif.). Dithiothreitol (DTT) and reduced glutathione (GSH) were obtained from Calbiochem Company (Los Angeles, Calif.).

A Technicon Amino Acid Analyzer was used with a 125 × 0.6 cm column of Chromobeads Type-A cation exchange resin in the sodium form (Technicon Corp., Ardsley, N.Y.). The column was maintained at 60° by a jacketed circulating water bath. Eluting buffers were pumped through the column at 0.6 ml per min. Ninhydrin reagent was obtained from Pierce Chemical Company (Rockford, Ill.). 0.01 *N* NaCN (0.2 ml/75 ml buffer) was added to the eluting buffers to reduce the ninhydrin, as described by ROSEN *et al.*[6]. Ninhydrin color of the effluent was measured at 570 mμ.

The first problem was to adapt the amino acid analyzer for the chromatography

TABLE I

COMPOSITION OF BUFFER GRADIENTS FOR PEPTIDE ELUTION

The pH of the solution in chamber 1 for Group I peptides was 4.5. The stock solutions of citrate buffer, pH 3.10, pH 5.10, and pH 10.50, were prepared as directed by the Technicon Corp.[7]. The stock solutions of buffers used for Group II peptides contained 10 ml Brij 35 per liter (Pierce Chemical Co.).

Autograd chamber No.	*Group I peptides*			*Group II peptides*		
	ml sodium citrate buffer			*ml sodium citrate buffer*		
	pH 3.10	*pH 5.10*	*pH 10.50*	*pH 3.10*	*pH 5.10*	*pH 10.50*
1	20	70		45		
2	20	70		45		
3		90		30	15	
4		40	50	10	35	
5			90		45	
6			90		20	25
7			90			45
8						45
9						45

of the peptides. Technicon Corp. recommended a gradient of pH 3.10–pH 10.50 for the eluting buffers using their nine-chamber Autograd device[7] for peptide elution. This gradient was modified in two ways for the chromatography of the cystine peptides as shown in Table I.

The peptides used were divided into two groups because of their chromatographic behavior and color yield with ninhydrin. L-Cystinyl-bis-(L-alanine) (Cys-Ala)$_2$, L-cystinyl-bis-(L-valine) (Cys-Val)$_2$ and L-cystinyl-bis-(L-leucine) (Cys-Leu)$_2$ made up

Group I. Group II consisted of bis-(L-alanyl-L-cysteinyl-L-tyrosine) (Ala-Cys-Tyr)$_2$, glycylglycyl-bis-cystine (Gly-Cys)$_2$, and oxidized glutathione (GSSG).

The elution gradient pH 4.5–pH 10.50 was used for the chromatography of Group I peptides. The molar color yields of the cystine dipeptides (Group I) were very low. Whereas 0.1–0.5 μmoles of most amino acids give sufficient color for automated analysis, 10 to 20 times these amounts of cystine dipeptides were necessary to give comparable color values. It is interesting to note that the amino acid cysteine has a ninhydrin color yield only 6% that of norleucine, while the color yield of cystine is about 50% that of norleucine[8]. Other dipeptides have ninhydrin color yields similar to those of amino acids[9,10].

The ninhydrin color yields of Group I peptides were increased by splitting the disulfide with $NaHSO_3$. The column effluent was mixed with $NaHSO_3$ in the analytical portion of the amino acid analyzer. $NaHSO_3$ (10 mg/ml) was dissolved in 4 *N* sodium acetate buffer pH 5.5 and pumped into the system at 0.1 ml per min (Fig. 1). After mixing with $NaHSO_3$, the effluent was mixed with ninhydrin solution in the normal manner. Absorption spectra of ninhydrin–peptide solutions showed peaks at 405 mμ and 570 mμ. Both peaks were approximately doubled if the peptide was reacted with bisulfite before ninhydrin color development. Since the amino acid analyzer is normally

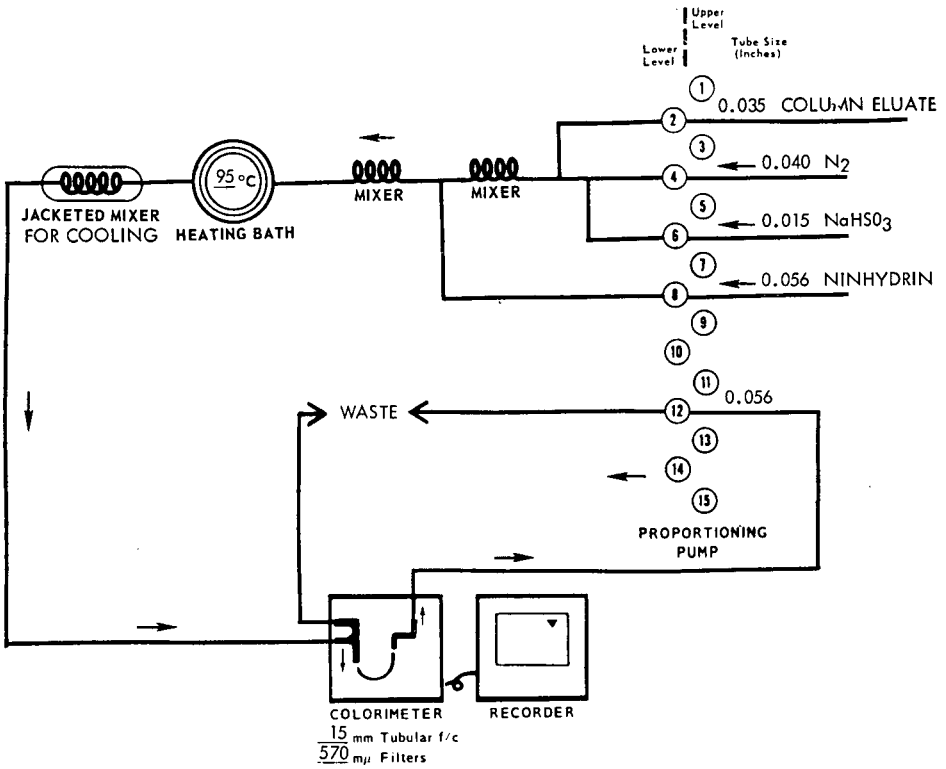

Fig. 1. Manifold arrangement of the Technicon Amino Acid Analyzer for the analysis of Group I peptides. Group II peptides were analyzed in the same system but without the addition of $NaHSO_3$.

equipped with a 570 mμ filter, we used this wavelength to measure color intensity.

A typical chromatogram of Group I peptides was carried out as follows. The column was washed with 0.2 *N* NaOH for 30 min and then regenerated with buffer of the same pH as at the start of the gradient (pH 4.5 for Group I). The regenerating buffer contained 0.01 *N* NaCN (0.2 ml/75 ml buffer). After the column had been equilibrated for 1.5 h, the sample was applied and the pH gradient elution started. A chromatogram of all Group I peptides was complete in 5 h. The mixed disulfide formed from a pair of dipeptides emerged between the elution peaks for each of the respective symmetrical disulfides.

Group II peptides, GSSG, $(Gly\text{-}Cys)_2$, and $(Ala\text{-}Cys\text{-}Tyr)_2$ had color yields close to those of amino acids and required a different pH gradient elution pattern. The buffer gradient for elution of Group II peptides was pH 3.10–pH 10.50 (Table I). Group II peptides gave much greater ninhydrin color yields than those of Group I and sulfitolysis of Group II peptides did not increase their color yields. Therefore, it was not necessary to react Group II peptides with $NaHSO_3$ in the amino acid analyzer.

The cystine peptides were prepared in the sulfhydryl form by reducing the disulfide bonds with dithiothreitol[11] at neutral pH. After 15 min, reduction of the peptides was complete and the pH was lowered to 2 to prevent reoxidation. The reduced peptides were separated from DTT on a 3.0 × 0.5 cm column of Dowex 50W X4 in the H^+ form. DTT was eluted with water and 6 *N* HCl was used to elute the reduced peptides. The eluates were neutralized and assayed for –SH content with ELLMAN's reagent (dithiobisnitrobenzoate, DTNB)[12]. The acid fractions containing the peptide were lyophilized to dryness, redissolved in water, and lyophilized again to remove excess HCl.

An aliquot of reduced peptide was adjusted to neutral pH and allowed to oxidize completely overnight. Complete oxidation was confirmed by a negative test for –SH groups with DTNB. The reoxidized peptide was then chromatographed in the appropriate system. From the ELLMAN test of the reduced peptide and the chromatogram of a reoxidized aliquot, the amount of reduced peptide recovered from the Dowex 50 column was determined. Each Group I peptide was reduced and isolated in this manner. The reduced peptides were divided into equal fractions (10 μmoles), lyophilized to dryness, and stored in a freezer.

Formation of mixed disulfides was the third problem. Group I and Group II peptides were treated separately because of their different color yields and chromatographic behavior. Equal fractions of Group I peptides were dissolved in 1.0 ml 0.1 *N* HCl, mixed together, and the pH adjusted to neutrality. The mixtures were allowed to oxidize overnight. Complete oxidation was confirmed by using DTNB to detect –SH groups. When oxidation was complete, each mixture was acidified with HCl, lyophilized to dryness, redissolved in 1 ml 0.1 *N* HCl, and then applied to the amino acid analyzer.

Group II peptides were treated differently. Reduced glutathione (GSH) was available commercially and was mixed individually with each of the other Group II peptides. Mixtures of GSH and the oxidized peptides were adjusted to neutral pH and thiol–disulfide interchange was allowed to occur until oxidation of all compounds was complete. The mixtures were acidified, lyophilized, redissolved in 0.1 *N* HCl, and chromatographed.

The areas under the ninhydrin peaks of all chromatograms were integrated with

a Technicon Integrator/Calculator. The amount of symmetrical disulfides were calculated based on color yields previously determined from known amounts of oxidized peptides.

RESULTS

The formation of mixed and symmetrical disulfides from reduced peptides appeared to occur randomly. Table II presents the amount of the various disulfides

TABLE II

PERCENT TOTAL S AS SYMMETRICAL OR MIXED DISULFIDES AFTER OXIDATION OF GROUP I PEPTIDES

Reduced peptides mixed together	*Oxidation products*					
	1 $(Cys\text{-}Ala)_2$	*2* *Cys–Ala* | *Cys–Val*	*3* $(Cys\text{–}Val)_2$	*4* *Cys–Ala* | *Cys–Leu*	*5* *Cys–Val* | *Cys–Leu*	*6* $(Cys\text{-}Leu)_2$
Cys–Ala + Cys–Val	23.4 24.1 (25) 23.6	47.8 49.7 (50) 51.4	28.8 25.6 (25) 26.0			
Cys–Ala + Cys–Leu	20.8 21.1 (25)			53.4 56.2 (50)		25.8 22.7 (25)
Cys–Val + Cys–Leu			22.9 22.9 (25) 19.2		50.5 51.1 (50) 52.2	26.6 26.0 (25) 28.6
Cys–Ala + Cys–Val + Cys–Leu	11.1 12.5 (11.1)	(22.2)	9.87 12.0 (11.1)	(22.2)	(22.2)	11.6 12.2 (11.1)

Numbers in parentheses are theoretical values for random combination. Experimental values for mixed disulfides were determined as the difference between 100% and the symmetrical disulfide values. A typical chromatogram is given in Fig. 2. Buffer gradients for elution are given in Table I. Other details of the chromatographic system are given in the text.

formed in each experiment with Group I peptides. The amount of a given sulfhydryl peptide (RSH) not represented in the symmetrical disulfide form (RSSR) was assumed to exist as half of a mixed disulfide (RSSR'). Thus, when RSH and R'SH were mixed in equal amounts and allowed to oxidize completely, the products were RSSR, R'SSR, and R'SSR', where R and R' designate peptides of differing amino acid composition. One would expect 50% of the total sulfur to appear as the mixed disulfide (RSSR') and 25% to appear in each of the two symmetrical disulfides, if RSH and R'SH combined randomly. As can be seen in Table II, the experimental results approach the theoretical values for random formation of disulfides.

Similarly, when all three sulfhydryl peptides were oxidized together, each of the resulting symmetrical disulfides contained close to the theoretical 11.1% (1/9) of the total sulfur (Table II). Fig. 2 is a typical chromatogram of Group I peptides. It is

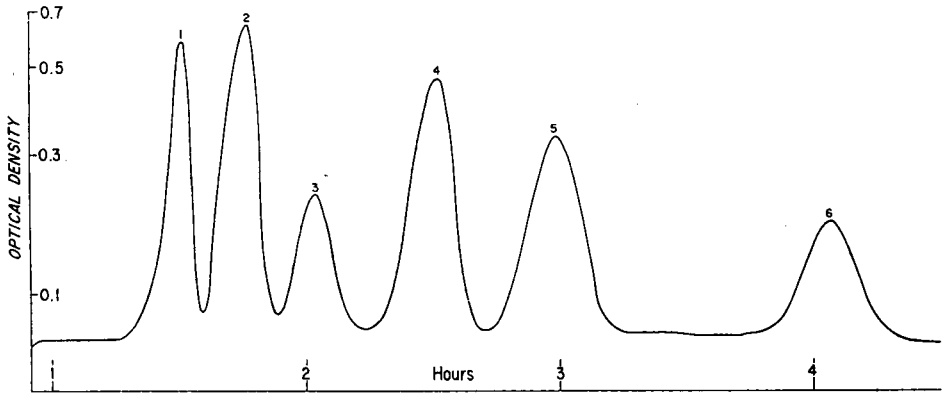

Fig. 2. A typical chromatogram of Group I peptides. The peaks are identified by the numbers given in Table II.

one of the experiments in which three reduced peptides oxidized to form three symmetrical and three unsymmetrical disulfides. When only two reduced peptides were oxidized together, the chromatograms were similar to Fig. 2 with peaks in the appropriate positions.

Since the interaction among Group I peptides appeared to occur randomly, the amino acid sequences of these peptides apparently have no influence on the formation of mixed disulfides.

The results of experiments with Group II peptides are presented in Table III.

TABLE III

PERCENT TOTAL S AS SYMMETRICAL OR MIXED DISULFIDES AFTER OXIDATION OF GROUP II PEPTIDES

Numbers in parentheses are theoretical values for random combination. Experimental values for mixed disulfides were determined as the difference between 100% and the symmetrical disulfide values. Buffer gradients for elution are given in Table I. Other details of the chromatographic systems are given in the text.

Peptides mixed together	*Oxidation products*					
	GSSG	*GS*	*GS*	*$(Gly–Cys)_2$*	*GS*	*$(Ala–Cys–Tyr)_2$*
		Gly–Cys	*Ala–Cys–Tyr*		*Ala–Cys–Tyr*	
GSH + $(Gly–Cys)_2$	23.5 25.5 (25)	51.9 50.6 (50)		24.6 23.7 (25)		
GSH + $(Ala–Cys–Tyr)_2$	24.8 (25)		52.7 (50)		22.5 (25)	
GSH + $(Gly–Cys)_2$ + $(Ala–Cys–Tyr)_2$	11.2 (11.1)	(22.2)	(22.2)	11.0 (11.1)	(22.2)	8.8 (11.1)

When GSH was mixed with either of the disulfides $(Gly\text{-}Cys)_2$ or $(Ala\text{-}Cys\text{-}Tyr)_2$, thiol–disulfide interchange appeared to occur randomly. Because random interaction was observed in this manner, it was not necessary to oxidize mixtures starting with all peptides in the reduced form.

When equal amounts of GSH and $(Gly\text{-}Cys)_2$ and $(Ala\text{-}Cys\text{-}Tyr)_2$ were mixed and oxidized, it appeared that equilibrium of thiol–disulfide interchanges was not reached. Therefore, the sulfhydryl forms of these peptides were mixed and allowed to oxidize. As can be seen in Table III, random formation of disulfides appeared to occur when all three Group II peptides oxidized in this manner. The tripeptide $(Ala\text{-}Cys\text{-}Tyr)_2$, was considerably less pure than the others. This may account for its greater deviation from the theoretical amount than the others.

DISCUSSION

The ninhydrin color yield of cystine is 50% that of normal amino acids and the color yield of cysteine is only 6%[8]. Ninhydrin color yields of Group I peptides (where cystine was in the N-terminal position) were all very low, similar to cysteine. Group II peptides (where cystine was in the middle or in the C-terminal position) all produced ninhydrin color yields similar to those of normal amino acids. Furthermore, sulfitolysis of N-terminal cystine peptides increased the color yields by a factor of about 2. It is apparent from these observations that the sulfur atoms of cystine have an effect on the reactivity of that compound's amino group toward ninhydrin.

Disulfide formation among the peptides studied appeared to occur randomly. The amino acid sequences did not influence the formation of any particular combination of peptides. These results are consistent with the concept that the amino acid sequences of a protein determines its conformation and the arrangement of disulfides between cysteine residues.

The chromatography of cystine peptides and the methods for preparing them in the reduced form may be of general usefulness in the future study of peptides and disulfides.

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STUDIES ON THE NERVE GROWTH FACTOR(NGF) FROM SNAKE VENOM MOLECULAR HETEROGENEITY

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SUMMARY

The nerve growth factor from the venoms of *Crotalus adamanteus* and *Bothrops jararaca* was analyzed by chromatography and gel filtration. The data obtained suggest that purified nerve growth factor aggregates and dissociates to form active molecules of various molecular weights. Evidence was found for the existence of an active nerve growth factor, possibly a fragment or a subunit, having a molecular weight of less than 5,000.

INTRODUCTION

Venoms from the three families of poisonous snakes have been shown to contain a specific protein factor endowed with a unique nerve-growth promoting activity[1–3]. Small amounts of crude venom (0.4–0.2 μg protein) added to the culture medium of explanted chicken embryonic sensory ganglia elicit a vigorous outgrowth of nerve fibers[2]. The venom NGF was identified with a protein molecule of approximately 20,000 molecular weight. Gel filtration studies[4], however, have shown that the NGF may be present in various venoms at different levels of complexity. In the present study, venoms of two species of *Crotalidae* were analyzed comparatively.

MATERIAL AND METHODS

Venom preparations

The Butantan Institute of Brazil kindly supplied the venom of *Bothrops jararaca*. *Crotalus adamanteus* venom was purchased from Sigma Chemical Co. (St. Louis). Various amounts of these dried venoms were weighed and dissolved in the desired buffer, maintaining the temperature from 0–4°. After centrifugation for 20 min at 10,000 r.p.m. in a Sorvall centrifuge at 0–4°, the active supernatants were used for chromatography.

Assays

Nerve-growth promoting activity was assayed in the fractions by the tissue-culture method devised by LEVI-MONTALCINI[5,6] using sensory ganglia from 8-day chick embryos. Series of five-fold dilutions were tested for each fraction. The dilution of the purified fractions was performed in physiological saline containing 2 mg/ml bovine serum albumin. The presence of inert proteins at the higher dilutions stabilized the NGF response. One Biological Unit was defined as the amount of each fraction necessary to produce a 3+ response *in vitro* (nerve fiber outgrowth).

Estero-protease activity was assayed by the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) obtained from Sigma Chemical Co.[7]. Incubations were carried out in Tris–HCl buffer, 50 m*M*, pH 8.0.

Proteins were measured by the method of LOWRY *et al.*[8] using bovine serum albumin as standard. Protein concentration in chromatographic and gel filtration experiments was approximated by measuring the optical density at 280 mμ using a Zeiss spectrophotometer.

Chromatography and gel filtration

Sephadex G-100 and G-75 medium grade, and Sephadex G-25 fine grade, were obtained from Pharmacia (Uppsala). Diethylaminoethyl cellulose (DEAE-cellulose) anion exchanger and carboxymethyl cellulose (CM-cellulose) cation exchanger were both of the microgranular, preswollen type of Whatman (DE 52, CM 52). Blue Dextran (Pharmacia) and purified cytochrome c, chymotrypsin, bovine serum albumin, and tyrosine from Sigma Chemical Co. were used to calibrate the gel filtration columns. Chromatography and gel filtration were carried out at 4°.

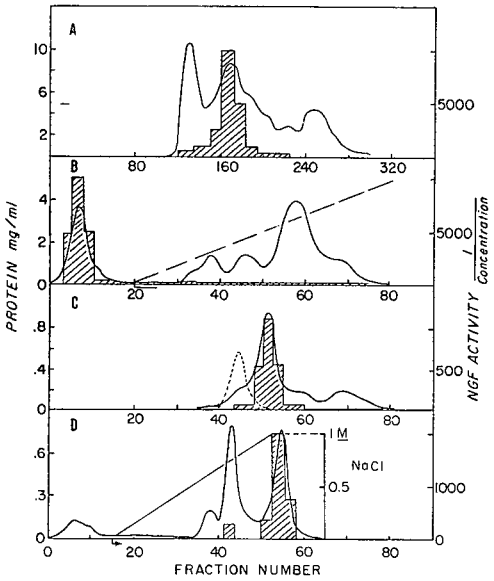
Electrophoresis and ultracentrifugation

Electrophoresis on polyacrylamide gel was carried out by the method of DAVIES[9] and ORNSTEIN[10] with the modifications of REISFELD AND LEWIS[11] used for electrophoresis at pH 4.3. Gels were stained with Coomassie Brilliant Blue R250 at a 1% concentration in 12.5% trichloracetic acid. Ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 59,000 r.p.m.

RESULTS

NGF from Crotalus adamanteus venom

The procedure adopted to analyze the NGF from this venom is summarized in Fig. 1. The supernatant from 1 g of crude venom dissolved in 6 ml of 50 m*M* Tris–HCl buffer, pH 7.3, was directly applied to a large Sephadex G-100 column equilibrated with the solvent buffer. The most active fractions, eluted in a broad region from 60,000 to 20,000 molecular weight, were collected and applied to a DEAE column after dialysis against the starting buffer, 5 m*M* Tris–HCl, pH 7.3. After thorough washing with the starting buffer, a gradient of NaCl (from 0 to 1 *M*) was applied to the column. All activity was recovered in the first unabsorbed peak and therefore together with proteins having zero or positive charge under these conditions. The active DEAE fractions were pooled and concentrated by lyophilization, and then applied to a Sephadex G-75 column at pH 7.3. The NGF activity separated in this way from a shoulder of esterase activity and other components was equilibrated at pH 5.0 with



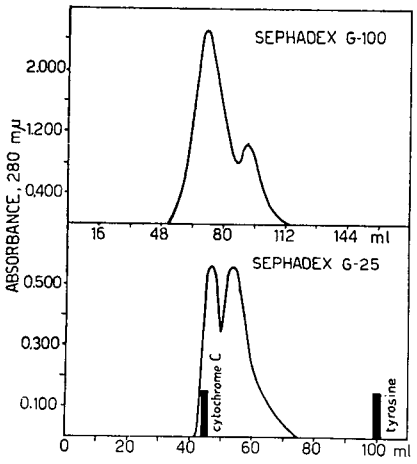


Fig. 1. Gel filtration and chromatography of NGF from *Crotalus adamanteus*. (A) Sephadex G-100 column, 115 × 3.5 cm, 50 m*M* Tris–HCl buffer, pH 7.3. (B) DEAE-cellulose column, pH 7.3. Dashed line indicates gradient of NaCl from 0 to 1 *N*. (C) Sephadex G-75 column, 100 × 1.5 cm, 50 m*M* Tris–HCl, pH 7.3. The dotted line indicates localization of esterase activity measured with BAEE as substrate. (D) CM-cellulose column, pH 5.0. Solid lines indicate protein concentration. Hatched areas localize NGF activity.

Fig. 2. Gel filtration on Sephadex G-100 and G-25 of purified *Crotalus adamanteus* NGF at pH 5.0. Bars indicate the elution volumes of cytochrome *c* and tyrosine.

acetate–acetic acid buffer, 50 m*M*. Chromatography on a carboxymethyl cellulose column was then performed using a gradient salt elution of 0 to 1 *M* NaCl. As shown in Fig. 1, most of the proteins were bound to the exchanger and were fractionated into a number of components. NGF activity was localized primarily in the third post-gradient peak, with a minor component of activity in the second.

This most active fraction was concentrated by lyophilization and applied to a Sephadex G-100 column equilibrated with acetate–acetic acid buffer, 50 m*M*, pH 5.0. As shown in Fig. 2, two main peaks appeared containing approximately equal amounts of NGF activity. The first, broad peak (A) was contained within elution volumes corresponding to a molecular weight of 30,000 to 20,000; whereas, the second component (B) was eluted in the range of 12,000 molecular weight. When this second peak of NGF activity was concentrated by lyophilization and reapplied on Sephadex G-25 column at pH 5.0, again two distinct components were obtained of approximately equal specific activity. The first was eluted in the exclusion volume and the second (C) was retarded. Ultracentrifugal analysis of components A and B gave $S_{20,w}$ values of 2.53*s* and 1.56*s* respectively (Fig. 3). As shown in Fig. 3, the sedimentation profile emerges as a single symmetrical peak, however, after 96 min the pattern of B begins to broaden and flatten assymetrically, that of A broadens, but remains symmetrical.

Another similar preparation when fractionated on Sephadex G-100 at pH 7.3, 50 m*M* Tris–HCl buffer, gave rise to three protein peaks having approximately the

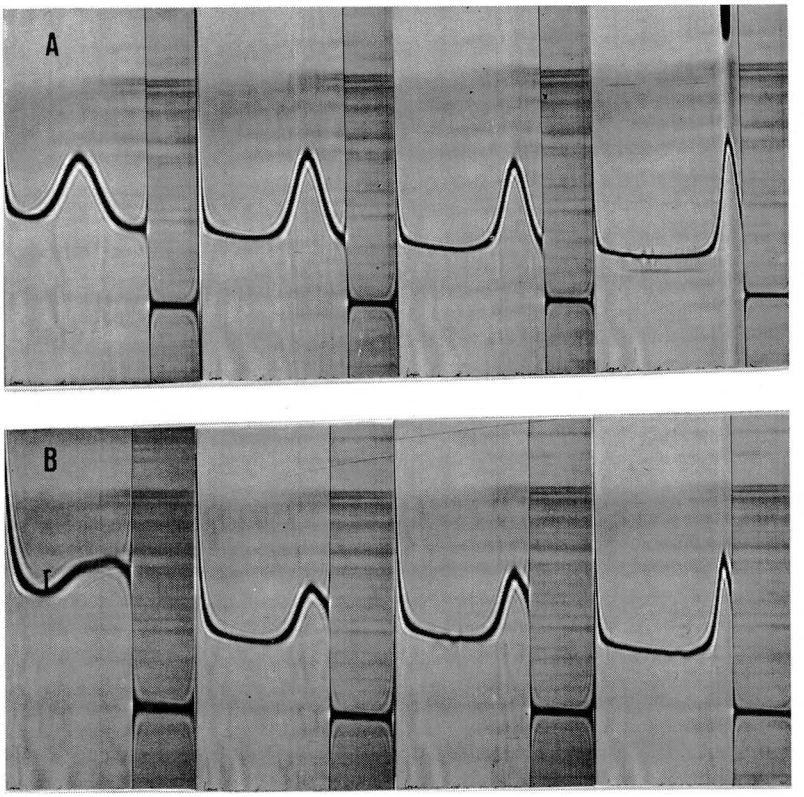


Fig. 3. Ultracentrifugation of NGF medium molecular weight components A and B (see text) from *Crotalus admanteus* venom. Runs were performed at a rotor speed of 59,000 r.p.m. Samples were dissolved in 100 m*M* acetate–acetic acid buffer pH 5.0. Photographs were taken every 16 min. The photographs from left to right are at times of 112, 64, 48, and 16 min, respectively.

same NGF specificity. As seen in Fig. 4, the first peak was excluded from the G-100 and is thus of molecular weight greater than 100,000. The second peak appeared at the same point as the A component of the other preparations, and had a similar $S_{20,w}$ of 2.56. The third broad peak D appeared later in the chromatogram as compared to B of the earlier preparations, and when rechromatographed on Sephadex G-25 appeared as a single, slightly retained peak.

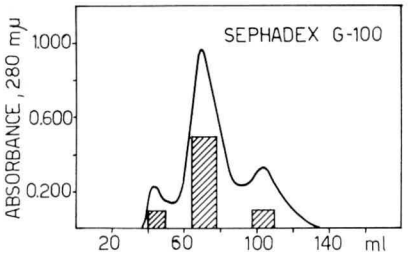


Fig. 4. Gel filtration of a purified *Crotalus adamanteus* NGF on Sephadex G-100 at pH 7.3. Hatched areas indicate NGF activity.

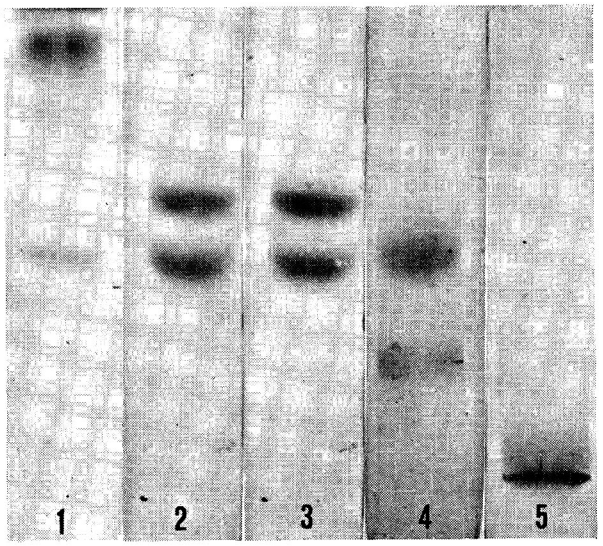


Fig. 5. Electrophoresis of various NGF components of *Crotalus adamanteus* on polyacrylamide gel at pH 4.3. (1) = Heavy molecular weight fraction; (2) = intermediate fraction A (see text); (3) = intermediate fraction B; (4) = low molecular weight fraction C; and (5) = low molecular weight component D.

The electrophoretic pattern at pH 4.3 on polyacrylamide gel of these different molecular forms of NGF obtained by gel filtration is shown in Fig. 5. As can be seen, A and B have identical patterns, each having two well-separated cathodic bands. The first excluded peak, however, contained only the more rapidly moving component plus several slowly migrating bands. The low molecular weight C fraction on the other band, had in common with the others only the faster migrating component, plus another more cathodic band. The low molecular weight fraction D exhibited only a single, very fast-moving component. When unstained gels of components A, B, C, and D were sliced and extracted with cold physiological saline, NGF activity could be recovered from the areas corresponding to each band, with no activity in other parts of the gel.

NGF from Bothrops jararaca venom

When one gram of venom from *Bothrops jararaca* was fractionated through Sephadex G-100 at pH 7.3 (Fig. 6), most of the NGF activity was recovered around the region of 40,000 molecular weight. The active fractions were pooled and applied to to DEAE column under the conditions described previously for the *C. adamanteus* venom. The major part of the activity was found in the first, unabsorbed peak. Some NGF activity under these conditions was, however, consistently found soon after the gradient began. Upon rechromatography of each peak using identical conditions, there was evidence of partial interconversion of one form into the other.

The pre-gradient peak was dialyzed against 50 m*M* acetate buffer pH 5.0 and applied to a CM-cellulose column. After initial washing with this starting buffer, a linear salt gradient from 0 to 1 *M* of NaCl was applied. As shown in Fig. 7, the activity was eluted in a rather broad peak. Esterase activity as measured by the hydrolysis of

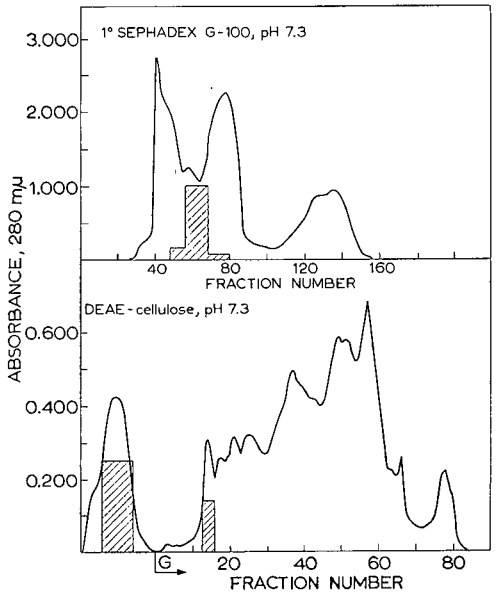


Fig. 6. Gel filtration and DEAE-cellulose chromatography of NGF from *Bothrops jararaca* venom. Hatched areas localize the NGF activity.

BAEE was also found in this peak, but could be separated from NGF upon rechromatography using a parabolic gradient from 0 to 0.5 *M* NaCl (pools 1 and 2). Successive rechromatography on CM-cellulose of both pools resulted in a further localization of the NGF activity. The most active fraction from both were finally pooled together and passed again over CM-cellulose. The activity was now recovered in one, sharp symmetrical peak. After concentration by lyophilization and subsequent dialysis, an analytical Sephadex G-100 chromatography (Fig. 7) revealed a pattern of protein and activity similar to that obtained for *Crotalus adamanteus*, the central peak having a molecular weight of approximately 30,000.

Similar results were obtained when the NGF fraction separated on DEAE-cellulose after the start of the gradient was applied on Sephadex G-100. Again NGF activity was found distributed in a broad area ranging from very high (greater than 100,000) to very low molecular weight forms. It is of interest that when the lowest molecular weight forms of NGF were pooled together and analyzed through a Sephadex G-25 column, they were found to have an elution volume sensibly greater than the void volume, and must therefore be of a molecular weight below 5,000.

DISCUSSION

LEVI-MONTALCINI first demonstrated that the nerve-growth-promoting activity elicited by some mouse tumors was due to a diffusable agent. In subsequent work, a nerve growth factor having similar biological properties was isolated in great quantities and identified as a protein molecule. The protein nature of the NGF was demonstrated by COHEN and repeatedly confirmed thereafter. The results of the present study

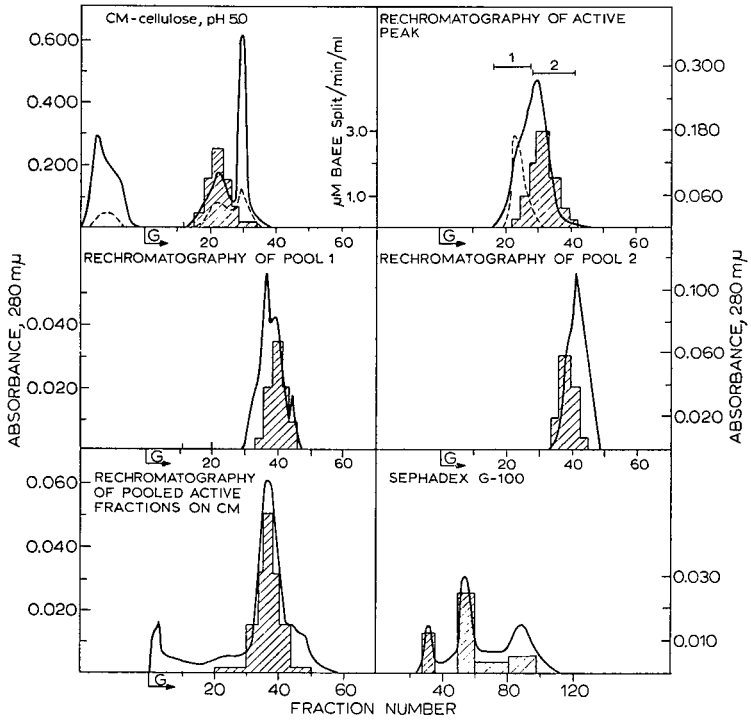


Fig. 7. Chromatography and rechromatography of *Bothrops jararaca* NGF. The first CM-cellulose chromatography was performed with a linear gradient of NaCl concentration from 0 to 1 *M* NaCl. Subsequent rechromatographies were performed on CM-cellulose columns using parabolic gradients of NaCl from 0 to 0.5 *M*. The Sephadex G-100 column was equilibrated with 50 m*M* Tris–HCl pH 7.3 + 0.100 *M* NaCl. Solid line, absorbance at 280 mμ. Dotted line, esteroprotease activity measured by hydrolysis of BAEE. Hatched areas, NGF activity.

demonstrated that snake venom NGF may exist in multiple molecular forms. Mild fractionation procedure such as gel filtration, revealed that there exists for each venom a predominant moiety of a given molecular weight value ranging in the species investigated from 60,000 to 20,000[4]. It should be pointed out that significant amounts of NGF activity are also found in other regions of the chromatogram corresponding to lower and higher molecular weights. The possibility that the various molecular forms of NGF could arise from an association–dissociation equilibrium of fundamental subunits rather than from unspecific interactions was investigated with purified preparations from *Crotalus adamanteus* and *Bothrops jararaca* venoms. In both cases there was evidence strongly in favor of this hypothesis. A highly purified and very active preparation from *Crotalus adamanteus* was resolved into two active components by gel filtration on Sephadex G-100. The second component undergoes further dissociation when processed on Sephadex G-25, giving rise to a form of molecular weight of approximately 5,000. Another purified preparation was resolved into three components on G-100, one of which was excluded from the column and therefore appears to be a large aggregate of NGF molecules. That two of the forms, with an $S_{20,w}$ of 2.53 and 1.56 respectively, had identical electrophoretic patterns consisting of two active

bands, could be attributed to the fact that each band represents a distinct active subunit or that each band represents a different state of aggregation of identical subunits.

The results obtained from *Bothrops jararaca* give evidence similarly for the existance of various molecular forms of NGF. Molecular heterogeneity was here evident from both chromatographic and gel filtration data. Two forms of NGF were in fact separated by DEAE chromatography. On rechromatography, each of these forms seemed to be moderately interconvertible to the other form. It is possible that this chromatographic behavior may result from the interaction of the NGF with other molecules, from conformational changes of the NGF, or from different states of aggregation of the NGF. One of these forms was further purified until it appeared chromatographically homogeneous. Nevertheless, this form was heterogeneous with respect to molecular weight when analyzed on Sephadex G-100, in a similar manner to the results obtained with *Crotalus adamenteus* NGF.

It is of interest that in both cases a fully active NGF of very low molecular weight, tentatively below 5,000, could be clearly demonstrated. This form may represent a fundamental NGF monomer or an NGF fragment produced during the various fractionation procedures, incomplete but still carrying biological activity. Immunochemical studies now in progress are expected to shed light on this problem.

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CHROM. 3642

THIN-LAYER CHROMATOGRAPHY FOR THE QUANTITATIVE SEPARATION OF QUINIDINE AND QUINIDINE METABOLITES FROM BIOLOGICAL FLUIDS AND TISSUES*

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SUMMARY

A chromatographic method for the study of quinidine metabolism is described. Quinidine, dihydroquinidine and metabolites were separated by thin-layer chromatography on Kieselgel G with methanol–acetone (4:1) as the solvent system. The fluorescing bands were divided into four main fractions and extracted with ethanol–acetone (1:1) from the adsorbent layer of the plate. The fluorescence of each fraction was measured with and without addition of acetic acid and sulfuric acid. A standard curve was used to transform fluorescence into μg quinidine base. The recovery from the plates varied between 75 and 95 per cent. The results obtained by this method were highly reproducible.

Amyl alcohol–benzene (1:1) was found optimal for the extraction of quinidine and its derivates from alkalized serum, urine and tissues. Removal of quenching substances and concentration of the quinidine bodies was achieved by transfer to sulfuric acid and second extraction with amyl alcohol–benzene. For further concentration, partial evaporation of the extraction solvent in a nitrogen atmosphere and at a temperature below 37° was used.

In recent years several methods have been published for the qualitative separation of chinchona alkaloids by thin-layer chromatography[1–3]. This paper describes in detail a method for the quantitative separation of quinidine, dihydroquinidine and their metabolites and the application of the method to the study of quinidine metabolism.

MATERIALS AND METHODS

Thin-layer chromatography

Adsorbent. Kieselgel G nach STAHL (Merck), pH 7.

* Supported by a grant from the Sigrid Juselius Foundation, Helsinki, Finland.

Solvents. Methanol p.a., acetone p.a., ethanol abs.

Procedure. Glass plates were coated with an adsorbent layer to a standard thickness of 0.5 mm using a Desaga applicator. The plates were air dried and activated for 2 h at 110°. Equally spaced vertical grooves were traced through the adsorbent layer to divide it into 2.5 cm wide strips. A horizontal groove was traced at the level selected for the solvent front at the end of the development. Quinidine bodies in amyl alcohol or in a mixture of amyl alcohol–benzene were applied to the starting line of each strip about 1.5 cm from the lower edge of the plate. The amount applied to a single strip was usually 1 to 5 μg and was determined exactly by applying a certain amount of solvent with a known quinidine concentration, using a Hamilton microsyringe. The chromatoplates were developed by the ascending technique in a rectangular chamber with ground lid at a temperature of 4°. The solvent system consisted of methanol and acetone (4:1). The time required was 40–50 min. The plates were dried under a stream of hot air and the positions of the fluorescent spots determined under U.V. light. Samples of commercial quinidine and dihydroquinidine were run for comparison. The strips were divided into fractions according to Fig. 1 and the adsorbent layer of each fraction was transferred quantitatively into a glass tube. After addition of 15 ml of a mixture of ethanol–acetone (1:1) the tube was shaken several times during 15 min and centrifuged. Five ml of the clear supernatant was transferred to another tube and the fluorescence was determined with and without addition of 2 drops of glacial acetic acid and 2 drops of 20% sulfuric acid[4,5] in a Beckman Ratio Fluorometer (phosphor sleeve position "360", primary filter UG 11, secondary filter Wratten 2 A). The apparatus was standardized, using 0.1 *N* sulfuric acid as a blank and 0.1 *N* sulfuric acid containing 0.1 μg quinidine base/ml to adjust fluorescence intensity by 50 or 100 on the scale. A standard curve obtained with quinidine in ethanol–acetone was used to convert the fluorescence into μg of quinidine base. The slope of the curve was checked each time with a known amount of quinidine in ethanol–acetone.

Extraction of quinidine derivates from biological fluids and tissues

Solvents. Amyl alcohol p.a., benzene p.a.

Procedure. To one part of serum or urine two parts of 0.1 *N* NaOH and 15 parts of a mixture of amyl alcohol and benzene (1:1) were added. The mixture was shaken vigorously for 5 min in a glass stoppered tube to transfer the quinidine bodies to the organic phase. After centrifugation for 10 min at 3000 r.p.m. part of the clear supernatant was used for the determination of quinidine, and the greater part was treated further for chromatography. Five ml was transferred to another tube and an equal amount of 0.1 *N* sulfuric acid was added. By shaking the tube vigorously quinidine bodies were transferred to the sulfuric acid, whereas quenching substances remained in the organic phase. Quinidine concentration was determined by measuring the fluorescence of the sulfuric acid. The remaining clear supernatant was transferred to another tube and a smaller amount of sulfuric acid was added to it to obtain concentration of quinidine bodies. The ratio of organic solvent to sulfuric acid had to be kept less than 15:1 to achieve complete recovery. After shaking the tube the sulfuric acid was transferred to another tube, made alkaline with 1 *N* NaOH, and an amount of amyl alcohol–benzene was added to give a ratio of organic solvent to inorganic phase of 2:1 at least. The tube was shaken and centrifuged as described. The organic phase

was transferred to another tube, and concentrated further by evaporation of the benzene in a nitrogen atmosphere. A small amount of the remaining solvent was added with a Hamilton microsyringe to 5 ml of 0.1 *N* sulfuric acid and its quinidine concentration was checked by determining the fluorescence. An amount of solvent calculated to contain 3.0 μg of quinidine bodies was pipetted on the starting line of the chromatoplate, but any amount between 1 and 5 μg gave good separation of fractions on the plate. Since urine contained relatively high amounts of quinidine bodies the first amyl alcohol–benzene extract could be used for chromatography.

Heart, liver, skeletal muscle etc. was cleaned of blood with dry filter paper and a small piece of tissue was taken for further analysis. About 1 g was found suitable, 0.2 to 0.5 g in the case of heart muscle. The tissue and 5 ml of physiological saline were ground in a Ultra-Turrax homogenizer to a homogenous suspension. The glass tube was weighed both empty and with its contents before and after homogenization. The losses due to suspension adhering to the grinding rod varied from 1 to 4% and were taken into account for the calculation of quinidine concentration. One ml of 1 *N* NaOH was added to the suspension and 30 ml of the mixture of amyl alcohol–benzene. The tube was shaken vigorously for 5 min and its contents treated further as described for serum and urine extracts.

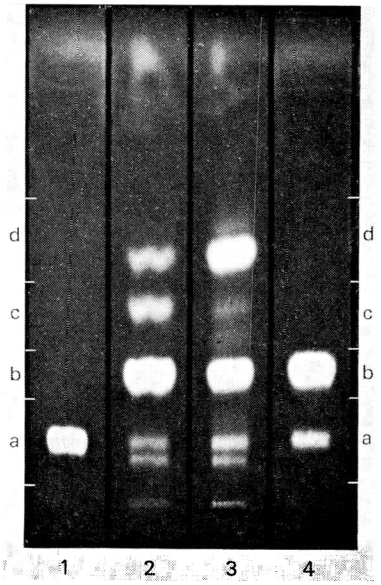

Fig. 1. Separation of quinidine, dihydroquinidine and metabolites by thin-layer chromatography. 1 = Dihydroquinidine, 2 = serum extract, 3 = urine extract, 4 = commercial quinidine contaminated with dihydroquinidine. Division into fractions which were extracted from the adsorbent layer is marked with *a*, *b*, *c*, and *d*. The plate was sprayed with sulfuric acid before U.V.-photography.

RESULTS

The separation of quinidine and quinidine derivates of serum and urine by thin-layer chromatography is shown in Fig. 1. For quantitative analysis the fluorescing

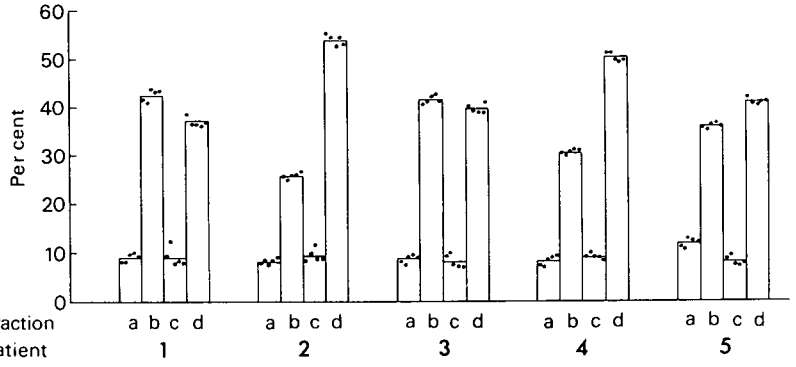

Fig. 2. Amyl alcohol–benzene extracts of five patients' urines separated into four main fractions by chromatography. The dots of each column represent values from five different plates.

bands of each strip were divided into four main fractions. Fraction *a* apparently contained dihydroquinidine and its metabolites; fraction *b* pure quinidine; fraction *c* the main quinidine metabolite circulating in blood and dihydroquinidine metabolites; and fraction *d* the main quinidine metabolite excreted into urine and a smaller amount of another quinidine metabolite. In addition, small amounts of several other metabolites were constantly found, and were located in the area of the fractions *a*, *c* or *d*.

In order to study the reproducibility of the chromatographic method, extracts of five different patients' urines were separated on five different chromatoplates each. The results are shown in Fig. 2. Fraction *c* showed the greatest relative variation, probably due to the difficulty of drawing a clear line between the fractions *b* and *c*, but even this variation was quite small. The total recovery from the plates varied

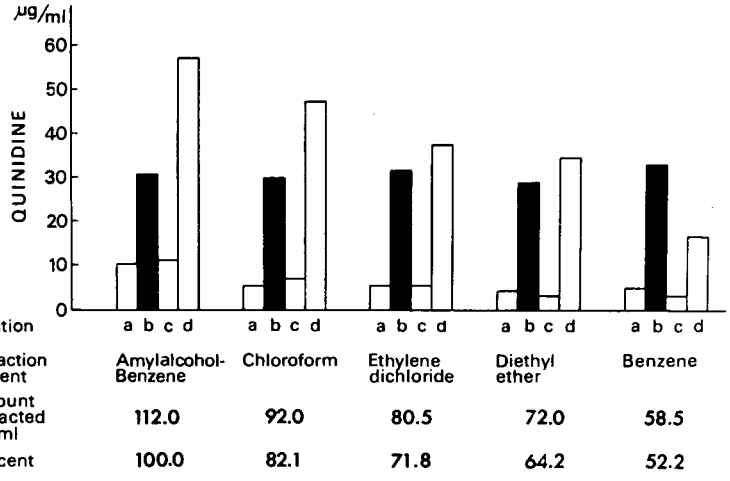

Fig. 3. Effect of different solvents on the extraction of quinidine, dihydroquinidine, and metabolites from urine. Fraction *b* (black column) represents quinidine which was extracted quantitatively by all five solvents. The amount of metabolites extracted decreased with diminishing polarity of the solvent.

between 75 and 95%. Within these limits recovery did not influence the relative distribution of the four fractions. The total recovery decreased with the increasing age of the plates after coating or after development. Fresh plates were therefore made every week and the fractions were always extracted a few hours after the run. For commercial quinidine the recovery was about 5% better than for extracted samples. The recovery of purified quinidine, dihydroquinidine and the main metabolite of quinidine was found to be equal from the same chromatoplate. It made no difference if the samples were run separately or after mixing them. Therefore the relative amount of each fraction was calculated as a percentage of the total amount recovered.

The importance of the right solvent for the extraction of quinidine bodies from biological fluids is visualized in Fig. 3. The total amount extracted decreased with the diminishing polarity of the solvent. However, the amount of pure quinidine was the same independent of the solvent. The mixture of amyl alcohol–benzene was superior to all other solvents used because even more polar quinidine derivatives were extracted quantitatively by this system. The amount of fluorescing compounds in the residue after extraction with amyl alcohol–benzene was found to be negligible.

DISCUSSION

For the separation of chinchona alkaloids on an adsorbent layer of Kieselgel G, a solvent system containing diethylamine has been found to give optimum conditions by several authors[1–3]. The aim of the present study was to find a solvent system suitable for the study of quinidine metabolites during quinidine medication. As known, commercial quinidine preparations are usually contaminated with 10 to 15% of dihydroquinidine. A solvent system containing methanol and acetone (4:1) was found to give good and highly reproducible separation of quinidine, dihydroquinidine and their main metabolites. The addition of diethylamine was not necessary. Development of the plates at low temperature improved the quality of separation. The system is not suited for separating stereo-isomers like quinine from quinidine.

Quinidine is metabolized in the human organism by hydroxylation to more polar derivates[6]. As shown by BRODIE *et al.*[7] the amount of quinidine bodies extracted from biological fluids depends on the polarity of the solvent used. A mixture of amyl alcohol and benzene was found most satisfactory in this study. Other solvents examined were in order of diminishing polarity: chloroform, ethylene dichloride, diethyl ether, and benzene. The amount of quinidine bodies extracted decreased in the same order. As shown in Fig. 3 the differences were caused by partial extraction of metabolites, whereas pure quinidine was extracted quantitatively by all solvents. This explains the differences in serum quinidine concentrations obtained by different methods in spite of the excellent recovery of quinidine by each method[8,9]. The extraction method used in this study was a modification of the double extraction method of CRAMÉR AND ISAKSSON[9], who used benzene as extraction fluid for the determination of serum quinidine.

Concentration of quinidine bodies for application on the chromatoplate was achieved by varying the ratio organic–inorganic solvent and by partial evaporation of the second amyl alcohol–benzene extract. During evaporation a temperature below 37° and a nitrogen atmosphere was found necessary to avoid changes in the relative distribution of the quinidine fractions. A second transfer to sulfuric acid was obligatory

in the case of serum and tissue extracts to remove lipids and quenching substances. In the case of urine, the first amyl alcohol–benzene extract could be used for chromatography, as the quinidine concentration is relatively high. Transfer to sulfuric acid, to amyl alcohol–benzene, evaporation of the amyl alcohol–benzene mixture, or extraction from protein containing fluids did not change the relative distribution of quinidine and its metabolites.

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CHROM. 3625

SEPARATION OF BILE PIGMENTS BY THIN LAYER CHROMATOGRAPHY*

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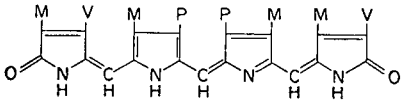
SUMMARY

Separation of bile pigments by ascending thin layer chromatography is described. Unesterified pigments separate on polyamide layer with methanol–water (3:1), the esterified ones on Silica Gel G with benzene–ethanol (100:8). Pigments inseparable in the above systems are separated: free violins and verdins on polyamide TLC with methanol–10% ammonia–water (9:1:2), esterified urobilin and stercobilin on Silica Gel G with chloroform–ethanol (100:2).

Bile pigments are open chain tetrapyrryl compounds[1,2] of which many have been isolated in nature or synthesized[1,3–5]. All, with very minor exception, are of IX, α configuration[6,7]. The parent bile pigment, biliverdin (I), is a totally unsaturated triene; hydrogenation in varying degree and position[2] is responsible for differing characteristics including chromatographic behavior. All members of the bile pigment family possess two carboxylic groups and four nitrogens of pyrrolenine (—N=) and/or pyrroline (—NH—) type[8]. For convenience we shall use the generally accepted common names as the corresponding systematic names are too cumbersome[2,5].



I

Thin layer chromatography (TLC) has but recently been employed for bile pigments, the methods thus far used being designed for identification or purification of specific compounds such as phycocyanobilin[9–11], phycoerythrobilin[12], or aplysioviolin[13]. These were separated on TLC in the form of dimethyl esters and comparatively large quantities have been used[9,12,13].

* Aided by grants from the National Science Foundation (GB 5578X), U.S. Public Health Service (AM 10539), and the Louis Weinberg Memorial Fund.

Most naturally occurring bile pigments are isolated as the free base or hydrochloride, hence a TLC method for separation in this form would be advantageous. Also, we have found that changes may be induced on esterification which in part counterbalance the advantages of working with esters. In the present study, the existing TLC methods have been examined and modified for application to most of the bile pigments. In addition, certain new methods have been devised. Both hydrochlorides and methyl esters have been used.

EXPERIMENTAL

Materials

Silica Gel G, from E. Merck, AG, Darmstadt, Germany.

Polyamide ITLC, from Gallard-Schlesinger Chemical Manufacturing Corp., 15 × 15 cm, coated on both sides.

Chloroform, ethanol, benzene, methanol, ammonia—analytical grade reagents.

Water—deionized and distilled.

METHODS AND RESULTS

Preparation of TLC plates

For preparation of 5 plates 20 × 20 cm, with 0.25 mm layer thickness, 30 g of Silica Gel G were placed in an Erlenmeyer flask to which 40 ml of distilled water was added with constant stirring until the slurry had a uniform consistency and was free of air bubbles. An additional 20 ml of water was then added with further stirring. The slurry is at once placed into a Desaga applicator adjusted for 0.25 mm layer thickness for even distribution over the plates. When thicker layers are desired, the amounts are increased proportionately. The plates are allowed to dry for 2 h in the air after which their treatment varies according to the solvent system to be used. For benzene–ethanol the plates are activated for 1 h at 120°, then stored in a desiccator over calcium chloride; for chloroform–ethanol, the plates are allowed to dry in the air for 24 h and are then ready for use, or may be stored in a desiccator over calcium chloride.

Esterification of bile pigments

Three methods were studied, *i.e.*, methanol–sulphuric acid (4:1, v/v)[1], diazomethane[1] and methanolic boron trifluoride[9]. The last is preferred (see below).

Separation of esters from the free pigments

In the following methods for separation of bile pigment esters on Silica Gel G, free bile pigments remain at the origin. This may be used for separating esters from unesterified pigments. Esters must be removed in advance of the separation of free bile pigments on polyamide TLC. This is done by dissolving the total pigments in chloroform and extracting unesterified pigments with 10% ammonia. The latter is acidified to Congo red paper with 7.5 *N* hydrochloric acid and extracted with chloroform. This is washed with water, dried by filtration and after suitable concentration is ready for chromatography.

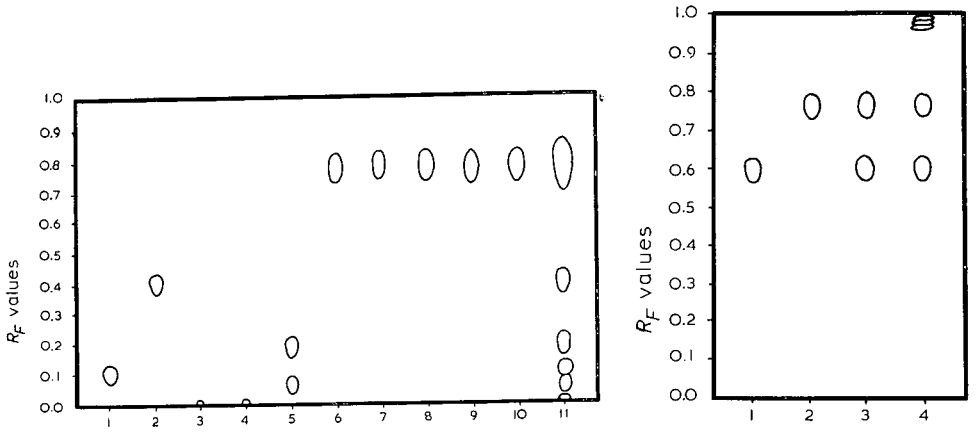


Fig. 1. TLC on polyamide layer developed with methanol–water (3:1, v/v). Spots correspond to: (1) Biliverdin; (2) mesobiliverdin; (3) bilirubin; (4) mesobilirubin; (5) mesobiliviolin[17], (a) stable, (b) labile; (6) *i*-urobilin, synth; (7) *d*-urobilin; (8) half-stercobilin; (9) stercobilin, nat.; (10) stercobilin, synth.; (11) mixture of 1–10.

Fig. 2. TLC on polyamide layer developed with methanol–10% ammonia–water (9:1:2, v/v/v), to 7 cm high. Spots correspond to: (1) Bilirubin; (2) mesobilirubin; (3) mixture of 1 and 2; (4) mesobiliviolins, mesobiliverdin, urobilin, mesobilirubin and bilirubin (from above downward).

Application

Ten μl of chloroform solution were applied to a spot. The total amount of pigment applied should vary from 0.1–5 μg; smaller amounts are undetectable without a densitometer, larger quantities cause tailing. For mixtures about 2 μg of each pigment were applied.

Separation of free bile pigments on polyamide layer

Unesterified bile pigments were separated on polyamide ITLC plates in Brinkmann 22 × 22 × 10 cm wide chromatography jars (15 × 15 cm plates, Fig. 1), or in cylindrical (2 in. I.D.) chromatography jars (5 × 15 cm plates, Fig. 2). The pigments were applied, the sheets were saturated for 45 min and ascending chromatograms were developed for about 2 h (until the solvent front moved 10 cm from the origin). The solvent was a mixture of methanol–water (3:1, v/v). Reference pigments and their mixtures were spotted 2 cm from the bottom of the sheet. For identification each plate with the unknown pigment was spotted in parallel and in mixture with the appropriate authentic reference compounds. In this system bile pigments forming hydrochlorides, *i.e.*, bilenes, bilitrienes and some bilidienes, were separated. Bilirubin and mesobilirubin are left at the origin. The runs required considerable time but the separation of the commonly occurring pigments, such as urobilins, violins, purpurins, verdins and rubins, was very satisfactory (see below). After development, the sheets were dried and the colored spots located. The bile pigments separated are shown in Fig. 1. The saturation period greatly influenced the separation. A comparison was made of saturation for 0, 15, 30, 45, 60 and 75 min. The best results corresponded to the longest saturation period. However, 45 min saturation gave satisfactory results and even shorter periods can be used for specific purposes, *e.g.*, mesobiliviolins could be separated satisfactorily without preliminary saturation.

The development time or origin–front distance may vary according to the aim. In special cases when separation of any two pigments is desired, *e.g.*, mesobiliviolin and mesobiliverdin, the front might be allowed to run further than 100 mm. When the objective was a separation of urobilinoids from violins or glaucobilin, runs of 60 mm were sufficient.

Bilirubin and mesobilirubin remained at the origin in the above system but were readily separated on the same polyamide ITLC sheet with methanol–10% ammonia–water (9:1:2, v/v/v). Other conditions were the same as already given. The results are shown in Fig. 2. The quality of separation depends on the distance from origin to front at the end of the run. Studies were made as to effect of the origin–front distance, 50, 55, 60, 75 and 100 mm, the best separation being observed at 55 mm. Other pigments such as violins, verdins and urobilinoids do not interfere with separation of mesobilirubin and bilirubin; all of the former group move almost with the solvent front (see run 4 in Fig. 2).

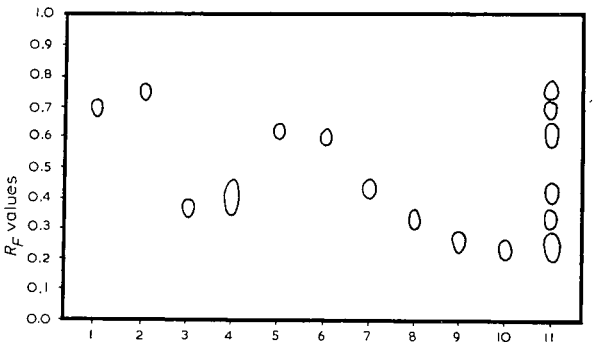


Fig. 3. TLC on Silica Gel G developed with benzene–ethanol (100:8, v/v), to 10 cm high. Spots correspond to: (1) Biliverdin; (2) mesobiliverdin; (3) bilirubin; (4) mesobilirubin; (5) bilipurpurin; (6) mesobilipurpurin; (7) labile mesobiliviolin; (8) stable mesobiliviolin; (9) urobilin; (10) stercobilin; (11) mixture of 1, 2, 5, 6, 7, 8, 9, 10.

TLC separation of dimethyl esters of bile pigments

This separation was achieved on a thin layer of Silica Gel G with solvent system benzene–abs. ethanol, (100:8, v/v) (Fig. 3). Other ratios of benzene–ethanol, 100:1, 100:4, 100:6, 100:8, 100:10, 100:12, 100:14 and 100:20 were compared and 100:8 was found to be the best for the general separation of bile pigments. The separating quality also depends greatly on the layer thickness and in this presentation 0.25 mm was used unless otherwise stated. For preparative separation with the layer 1 mm thick, better results were achieved with a slightly higher proportion of ethanol in the mixture, *i.e.*, 100:10 to 100:14. All bile pigments are separable in this system except bilenes, *e.g.*, urobilins and stercobilins, which move as one spot. TLC plates were developed in Brinkmann 22 × 22 × 10 cm wide chromatography jars. They were saturated for 30 min and developed until the solvent front reached 10 cm from the origins (these were 2 cm from the plate bottom), on an average of about 30 min. Drawings of developed chromatograms are shown in Fig. 3.

Urobilin and stercobilin were separated on Silica Gel G on 20 × 20 cm plates

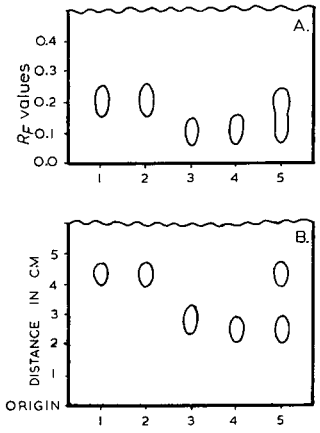


Fig. 4. TLC on Silica Gel G, developed with chloroform–ethanol (100:2, v/v). (A) Developed to 10 cm high, taken out, dried and developed again 5 times. (B) Developed to 10 cm high and allowed to run for another 3 h. Spots correspond to: (1) *i*-Urobilin, synth.; (2) *i*-urobilin, nat.; (3) half-stercobilin; (4) stercobilin, nat.; (5) mixture of 2 and 4.

with chloroform–ethanol (100:2, v/v) as the solvent. Plates were saturated for 30 min, then run until the front reached 10 cm above the origin. They were then taken out and dried. This process was repeated five times. The separation is shown in Fig. 4A. Better results were obtained when the plate was left in the developing chamber and the front was allowed to run to the top of the plate and then left in the chamber for another 3 h. In this case R_F values cannot be calculated but separation is greatly improved (Fig. 4B); the numerals on the axis indicate distances from the origin in centimeters. Both methods limit the separation according to saturation of the end rings, the di-pyrrolinones, with both unsaturated, including the natural *i*- and *d*-urobilins being separated from the di-pyrrolidones, both end rings saturated (natural and synthetic stercobilin), and the pyrrolinone-pyrrolidones with one end ring saturated, represented here by half-stercobilin[15]. Stercobilin and half-stercobilin travel as a single spot in this system in contrast to the dipyrrolinones.

Visualization of the pigments

Bile pigments are strongly colored compounds and they are visible on the thin layer plates without special treatment. The amount of the pigment which can be detected with the naked eye varies according to pigment color and lies in the region of 1–5 μg. Smaller quantities (0.1–1.0 μg) can be detected under U.V. light since most bile pigments fluoresce. Those not fluorescing have fluorescing metal complexes, thus after spraying with zinc acetate in ethanol, they can be detected under U.V. light. With these methods of visualization the bile pigments are readily detected.

DISCUSSION

With certain exceptions the above described chromatographic methods permit separation and identification of the components of mixtures of bile pigments. Separation is very convenient for most of the free (unesterified) bile pigments and by using this

method, loss incident to esterification is avoided. If preparation of the pigments excluded their esterification they can be applied directly; however, with any possibility of esterification, esters have to be removed or erroneous results will be observed. Urobilins and stercobilins (bilenes) run on polyamide layer as one spot. They can be separated from all other bile pigments but are not differentiated. This is not surprising since the number of carboxylic groups and pyrrolenine-pyrroline nitrogens are the same for both compounds. Rubins, *i.e.*, mesobilirubin and bilirubin, are not separated with methanol–water (3:1) and must be removed before chromatography in order to avoid tailing. They are easily separated with methanol–10% ammonia–water (9:1:2). Esters of bile pigments often allow better separation since the predominant carboxylic groups are blocked. Consequently the separation depends on whether the nitrogens are —N= or —N—. These differ between various pigments because the variation in this respect relates to the number of conjugated double bonds. This influence is much more important than the strongly polar carboxyl groups which are identical in all bile pigments.

There is only one group of pigments, *i.e.*, bilenes, which are not separated on Silica Gel G with benzene–ethanol (100:8). Bilenes consist of both natural and synthetic urobilins, stercobilins, half-stercobilin and their stereoisomers. Dipyrrolinones, such as the *d*- and *i*-urobilins, are separated from di-pyrrolidones, such as stercobilin, or pyrrolinone-pyrrolidone, such as half-stercobilin (see above) with chloroform–ethanol (100:2); however, *d*- and *i*-urobilins are indistinguishable in this system; the same is true for stercobilin and half-stercobilin. Further modification of this method is being studied and shows promise of separating the stereoisomers of the urobilins and stercobilins[16].

The benzene–ethanol (100:8) system can be changed according to the objective. For thicker layers of Silica Gel G, more polar, *i.e.*, more ethanol containing systems should be used. For example, this may be necessary in cases of marked predominance of a single pigment. Separation of methyl esters is advantageous except for adverse effect of esterification on the pigments. The most commonly used method of esterification with methanol–hydrochloric acid or sulphuric acid is too strenuous for bile pigments and some oxidation products are formed during the procedure[14]. Diazomethane is better since no oxidation products are formed; however, small amounts of unidentified products have been noted. Boron trifluoride is preferred since it is more protective.

A word of caution must be added. Esters of bile pigments tend to change on standing in chloroform. The proportion of pigment remaining at the origin increases and additional spots appear. For example, biliverdin and mesobiliverdin exhibit spots corresponding to purpurins; urobilins give spots corresponding to violins and glaucobilin. In order to prevent this change before chromatography it is necessary to protect the chloroform solution and use it as soon as possible after preparation. If dissolved at once before TLC and the remaining solution evaporated to dryness, the pigment can be stored for months without noticeable change.

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CHROM. 3650

DETERMINATION OF THE 2,4-DINITROPHENYLHYDRAZONES OF URINE KETOSTEROIDS BY THIN LAYER DENSITOMETRY

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SUMMARY

A double beam scanning spectrodensitometer is used for the determination of DHEA (dehydroepiandrosterone = 3β-hydroxyandrost-5-en-17-one), androsterone (3α-hydroxy-5α-androstan-17-one) and etiocholanolone (3α-hydroxy-5β-androstan-17-one) from urine. After ether solvolysis, purification and reaction with 2,4-dinitrophenylhydrazine, aliquots are developed on thin layer plates and quantitated directly.

The colorimetric determination of the 2,4-dinitrophenylhydrazones of ketosteroids from biological material has been reported recently[1-3]. The excellent separation behavior of these derivatives on thin layer plates and their high molar extinction (ε for 17-ketosteroids = 23,600) made a further simplification of the method possible. Instead of eluting the hydrazones and quantitating them in a spectrophotometer, they could be determined directly on the plate with a new instrument, a double beam scanning spectrodensitometer.

METHODS

Performance of the reaction

Five ml urine is acidified with 5 *N* HCl to pH 1, saturated with ammonium sulfate and extracted with 2 × 20 ml ethylacetate. The extract is neutralized with 0.5 ml ammonia and evaporated. The residue is shaken in 40 ml of 1% perchloric acid in ether and incubated for at least 15 h at 39–40°. A short boiling should precede the tight stoppering of the tube. The ether is then washed with 5 ml of 5 *N* NaOH and 3 × 5 ml water, dried over sodium sulfate and evaporated.

For the reaction itself, 0.1 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in ethylacetate is added and the solvent is evaporated. The residue is dissolved in 1 ml of 0.03% (w/v) trichloroacetic acid in absolute benzene and kept for 40 min at 40°. After this the solution is filled up with benzene to the original urine volume, so that certain aliquots can be withdrawn easily.

* Ford Fellow in Reproductive Biology.

Chromatography

For chromatography Silica Gel G plates of 250 μ thickness without binder are used (Analtech, Wilmington, Del.). With a special scoring device (Schoeffel Instrument Corp., Westwood, N.J.) the thin layer surface is divided into bands of 1 cm width. For duplicate determination dried 0.1 and 0.2 ml aliquots of the reacted urine are transferred with 3 × 0.03 ml benzene to the plate using a 50 μl syringe. A 0.8 cm starting line is formed. Because of the double beam operating principle of the densitometer, only alternate bands are loaded. The blanks serve as reference for the instrument. On each plate standard steroid hydrazones are run as reference.

A rapid separation of dehydroepiandrosterone (DHEA), androsterone and etiocholanolone hydrazones can be achieved by chromatography of the aliquots in system A (chloroform–carbon tetrachloride, 2:1 v/v) followed by unidimensional chromatography of the same plate in system B (chloroform–dioxane, 94:6 v/v) (Fig. 5). A better purification and separation requires chromatography of the total urine residue in system B on a 5 cm plate. The zones of the steroid hydrazones are eluted with 3 × 3 ml chloroform. Aliquots corresponding to 0.5 and 1.0 ml urine are rechromatographed in system B on plates prepared for densitometry. Combined DHEA and androsterone hydrazones can run in one band (Fig. 6). Etiocholanolone is run in another band (Fig. 7).

Fig. 1. The densitometer (Schoeffel Instr. Co., Westwood, N.J., U.S.A.). Model SD 3000 (about $^1/_{10}$ of original size).

Quantitation

Direct quantitation of the plates (optimal wavelength = 367 mμ) is performed in a Schoeffel spectrodensitometer, Model SD 3000 (Fig. 1). An optical density computer, Model SDC 300, gives the data to an integrating 10″ strip recorder, Model SDR 303 (Schoeffel Inst. Corp., Westwood, N.J.). Peak areas are calculated by triangulation (height × width at $\frac{1}{2}$ height) and interpolated on standard curves (Figs. 2 and 3). A plate, once it contains 17-ketosteroid hydrazone standards, can be

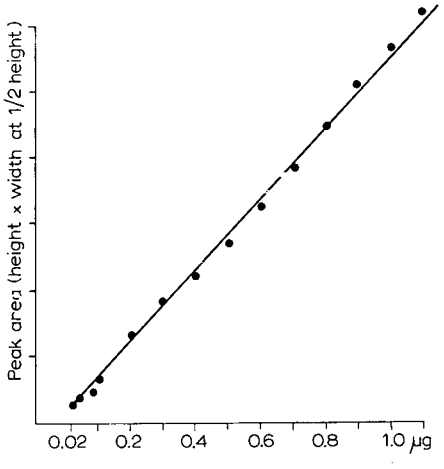

Fig. 2. Calibration curve of the densitometer (λ = 367 mμ) with recorder full scale at optical density = 0.5.

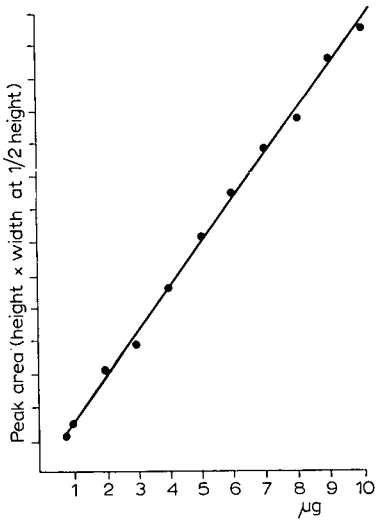

Fig. 3. Calibration curve of the densitometer with recorder full scale at optical density = 1.5.

used at any time for calibration of the instrument settings. The scanning of a 20-cm band by the densitometer takes about 20 sec.

Reliability

The reliability for the reaction of urine ketosteroids with 2,4-dinitrophenylhydrazone has been published in detail elsewhere[3]. The accuracy for DHEA sulfate, androsterone sulfate and androsterone glucuronoside from 10 ml urine was 91–95%. The precision ranged between $\pm$3.8 and $\pm$7.2%. Calculated from 6 blank determinations the sensitivity of the densitometric method ($= 6 \times S_{b1}$) lay near 0.05 μg 17-ketosteroid/sample. The sensitivity of the instrument ranged between 0.02 and 0.04 μg. Setting the recorder full scale at different optical density values, a good linearity was achieved between 0.02 and 10.0 μg (Figs. 2 and 3). The precision for one

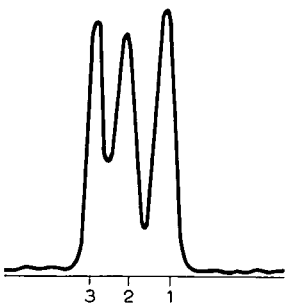

Fig. 4. Separation of about 2.5 μg each (1) DHEA, (2) etiocholanolone and (3) androsterone hydrazones in system B.

TABLE I

URINARY 17-KETOSTEROID 2,4-DINITROPHENYLHYDRAZONES DETERMINED BY EITHER (a) CONVENTIONAL SPECTROPHOTOMETRY OR (b) SPECTRODENSITOMETRY (μg STEROID/ml)

Urine No.	*Steroid*	*(a) 5 ml*[a]	*(a) 5 ml*[a]	*(a) mean*	*(b) 0.1 ml*[b]	*(b) 0.5 ml*[c]	*(b) 1.0 ml*[c]	*(b) mean*
1	D	2.78	2.70	2.74	2.20	2.50	3.05	2.58
	A	5.93	6.58	6.25	5.50	6.40	5.85	5.91
	E	3.60	4.83	4.21	3.85	4.00	4.10	3.98
2	D	3.78	4.16	3.97	3.55	3.70	3.85	3.70
	A	4.48	5.02	4.75	4.70	4.60	4.90	4.73
	E	4.36	5.04	4.70	4.05	4.45	4.55	4.35
3	D	6.20	6.28	6.24	6.55	5.95	6.10	6.20
	A	4.52	4.23	4.37	4.20	3.80	3.40	3.80
	E	2.87	2.88	2.87	3.20	2.18	2.60	2.66
4	D	3.45	3.28	3.36	2.95	3.35	3.20	3.20
	A	4.07	3.72	3.89	4.40	3.95	4.05	4.13
	E	2.58	2.41	2.49	2.80	2.70	2.65	2.71
5	D	3.37	3.69	3.53	3.10	3.25	3.45	3.26
	A	2.07	1.64	1.85	1.45	1.85	1.80	1.70
	E	2.89	2.17	2.53	1.95	2.60	2.45	2.33
6	D	2.40	2.95	2.67	2.05	2.70	2.85	2.53
	A	5.20	5.78	5.49	4.80	5.40	5.55	5.25
	E	4.28	4.07	4.17	3.80	4.35	4.25	4.13

[a] One chromatography in system B.
[b] Two unidimensional chromatographies on one plate in systems A and B.
[c] Chromatography in system B, elution and rechromatography in system B. D = dehydroepiandrosterone; A = androsterone; E = etiocholanolone.

peak in 4–8 replicate determinations was less than 0.5%. The specificity is ensured by solvent partition, at least two fold chromatography and by visualization of a proper curve.

RESULTS AND DISCUSSION

Table I gives total DHEA, androsterone and etiocholanolone (μg/ml) from urines of 6 healthy males (29–35 years of age). Two 5 ml aliquots were measured in a Beckman DK-2 spectrophotometer (a). A third 5 ml sample (b) was carried through the 2,4-dinitrophenylhydrazone reaction. Three aliquots (0.1, 0.5 and 1.0 ml) were quantitated in the spectrodensitometer, either after twofold unidimensional chromatography in systems A and B or after purification in system B, elution and rechromatography in system B. Figs. 5, 6 and 7 show corresponding curves (from urine No. 1 in Table I).

The values obtained by the densitometer corresponded well to those from a colorimetric method. For routine clinical analysis this could also be achieved when an 0.1 ml aliquot was developed on one plate without elution. Hence, a relatively simple and rapid method is offered. Ten urine samples can be finished easily in 24 h if they are incubated over night for solvolysis.

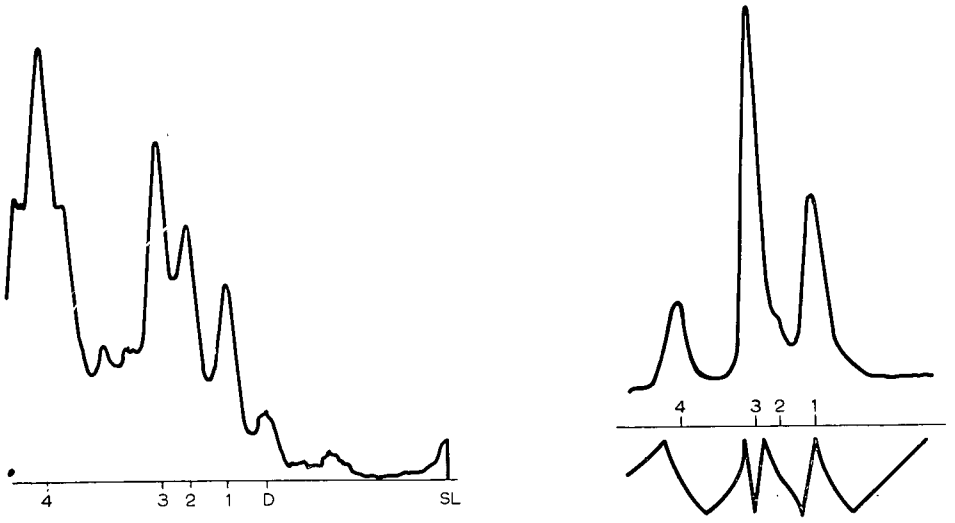

Fig. 5. 0.2 ml urine extract after reaction with 2,4-dinitrophenylhydrazone and unidimensional chromatography in systems A and B. (SL = starting line; D = reagents excess; 1 = DHEA; 2 = etiocholanolone; 3 = androsterone; 4 = nonpolar compounds).

Fig. 6. (1) DHEA, (2) rest of etiocholanolone and (3) androsterone hydrazones from 0.5 ml urine after chromatography in system B, elution and rechromatography in system B (lower curve = disc integrator curve).

A further decrease in time can be achieved when acid hydrolysis is introduced. For this purpose 5 ml of crude urine plus 0.5 ml of concentrated sulfuric acid are incubated for 1 h at 80°. The free steroids are then extracted with ether. Most of DHEA will be destroyed, yet androsterone and etiocholanolone from 5 urine samples can be determined in 4 h. The solvolysis of the steroid conjugates of urine in perchloric acid–ethyl acetate was found to be unreliable.

Several recent papers deal with the densitometry of steroids on sprayed thin layer plates[4–6]. In the present method stable color derivatives of ketosteroids are separated and quantitated as such by a practical and highly efficient and reliable instrument.

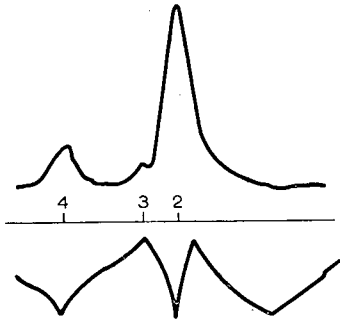

Fig. 7. (2) Etiocholanolone and (3) rest of antrosterone hydrazones from 0.5 ml of the same urine as in Fig. 6 after the same treatment (lower curve = disc integrator curve).

The method was also adapted for analysis of ketosteroids from plasma. This will be described elsewhere.

ACKNOWLEDGEMENTS

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CHROM. 3598

PAPER CHROMATOGRAPHIC CHARACTERISTICS OF SOME NEW 1-OXYGENATED STEROIDS

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SUMMARY

In an investigation of the paper chromatographic characteristics of a number of 1-oxygenated steroids, it has been shown that the contribution to polarity of the C-11 carbonyl group in 1,11-diketones is aberrantly large and that, contrary to the general rule, the equatorial member of most pairs of epimeric 1-ols is the more mobile. Various mechanisms have been proposed to account for these results.

INTRODUCTION

Recently we isolated a new 1-hydroxylated metabolite of cortisol, namely 1β, 3α,17α,20β,21-pentahydroxy-5β-pregnan-11-one and, in the course of its degradation, prepared a number of saturated and unsaturated 1-oxygenated 5β-pregnanes and androstanes both bearing and lacking carbonyl groups at C-11[1,2]. As illustrated in other publications from this laboratory[3–5], we made extensive use of partition paper and partition column chromatography to analyze the composition and to separate the components of reaction mixtures, to check the effectiveness of other fractionating procedures, and to verify the homogeniety of crystallized products. In addition to its general utility in this study, the method revealed aberrant paper chromatographic behavior in enough compounds to justify a more extended study, and to indicate the merits of correlating these results with certain unusual chemical properties of these compounds.

METHODS

With the exception of compound 29, the steroids used in this study were prepared previously[1,2,6] or are known substances. Compounds 30 and 31 were supplied by Drs. STEPHEN KRAYCHY, WALTER R. BENN and RAPHAEL PAPPO of G. D. Searle and Company. Compound 37 was prepared by incubating 5β-androstan-3α-ol-17-one with *Fusarium lini* (Bolley)[7] or, more successfully, with a *Penicillium* species (ATCC 11598). Its constitution was established by NMR spectroscopy.

Preparation of 5α-androstane-1β,3β-diol-17-one (compound 29)

To a solution of 16 mg of androst-5-ene-1β,3β-diol-17-one in ethanol–cyclohexane, 5% palladium on carbon was added and the suspension was agitated for 30 min at room temperature in a hydrogen atmosphere. The product was isolated in the usual way and twice crystallized from acetone–*n*-hexane. Constants: m.p. 199–199.5°, $[\alpha]_D$ + 72.9° (methanol). Its structure was established by sodium borohydride reduction to the known[8] 5α-androstane-1β,3β,17β-triol; the melting point of our product (220–222.5°) was found by Dr. BENN to be unaltered on admixture with the reference sample of 5α-androstane-1β,3β,17β-triol (m.p. 218.5–222°) prepared by Dr. PAPPO in the Searle laboratory. The infrared spectra of the two triols were identical.

As indicated in our cited publications, paper chromatography was carried out at 25 ± 1° after an equilibration period of not less than 8 h. Steroids were applied in the lowest discernible concentration. Detection methods included application of the Zimmermann reagent[9] (after *in situ* periodic acid oxidation in the case of 17,20,21-glycerols[10]), or dipping in 10% alcoholic phosphomolybdic acid followed by heating at 60–80°. The composition of systems referred to by number in Tables II–V appear in Table I. In those cases where we were interested in measuring the effects of individual

TABLE I

COMPOSITION OF PAPER CHROMATOGRAPHIC SYSTEMS

System No.	*Composition*[a]
1	Tol., 50; Iso., 150; AM, 150; HOH, 50 ml.
2	Tol., 170; Iso., 30; AM, 150; HOH, 50 ml.
3	Tol., 140; III-bu., 60; AM, 70; HOH, 80 ml.
4	EA, 30; Iso., 170; AM, 100; HOH, 100 ml.
5	EA, 75; Iso., 125; AM, 70; HOH, 130 ml.
6	Tol., 60; Iso., 140; AM, 160; HOH, 40 ml.
7	Bz., 5 ml, diluted to 25 ml with EA.
8	Tol., 140; Iso., 60; AM, 130; HOH, 70 ml.
9	Iso., 150; III-bu., 50; AM, 30; HOH, 170 ml.
10	EA, 50; Iso., 150; AM, 50; HOH, 150 ml.
11	IPE, 110; Hep., 90; AM, 140; HOH, 60 ml.
12	Tol., 105; EA, 95; AM, 120; HOH, 80 ml.
13	Tol., 145; III-bu., 55; AM, 70; HOH, 80 ml.
14	HOAc, 130; HOH, 70; Dodecane, 5 ml. Paper impregnated by drawing through a 15% (v/v) solution of dodecane in acetone. Immediate application of steroids and chromatography without prior equilibration.

[a] Tol. = toluene; Iso. = isooctane(2,2,4-trimethylpentane); AM = absolute methanol; III-bu. = *tert.*-butanol; EA = ethyl acetate; Bz. = benzene; HOAc = glacial acetic acid; IPE = isopropyl ether; Hep. = *n*-heptane.

functional groups on mobility, the results are expressed as ΔR_{Mg} values, a designation proposed by BUSH[11] based on the original ΔR_M concept of BATE-SMITH AND WESTALL[12].

RESULTS

Table II gives the ΔR_{Mg} values for the C-11 carbonyl group contribution in nine pairs of 5β-androstanes or androstenes and two pairs of 5β-pregnanes chromatographed

TABLE II

CONTRIBUTION OF C-11 CARBONYL GROUP IN 5β-ANDROSTANES, ANDROSTENES AND 5β-PREGNANES

Compound No.	*Compound*	*Pair*	*System*	R_F	ΔR_{Mg}	*System*	R_F	ΔR_{Mg}
1	5β-Androstane-3,17-dione	1	1	0.79	0.75	4	0.82	0.73
2	5β-Androstane-3,11,17-trione			0.40			0.46	
3	5β-Androstane-1,17-dione	2		0.87	1.46		0.89	1.54
4	5β-Androstane-1,11,17-trione			0.19			0.19	
5	5β-Androst-1-ene-3,17-dione	3		0.72	0.61		0.80	0.78
6	5β-Androst-1-ene-3,11,17-trione			0.39			0.40	
7	5β-Androst-2-ene-1,17-dione	4		0.77	1.48		0.83	1.75
8	5β-Androst-2-ene-1,11,17-trione			0.09			0.08	
9	Androst-4-ene-3,17-dione	5		0.60	0.72		0.66	0.82
10	Androst-4-ene-3,11,17-trione			0.22			0.22	
11	5β-Androstan-3α-ol-17-one	6	2	0.86	0.43	5	0.91	0.47
12	5β-Androstan-3α-ol-11,17-dione			0.70			0.77	
13	5β-Androstan-3α-ol-1,17-dione	7		0.65	0.87		0.71	1.26
14	5β-Androstan-3α-ol-1,11,17-trione			0.20			0.12	
15	5β-Androstane-1β,3α-diol-17-one	8		0.24	0.43		0.38	0.69
16	5β-Androstane-1β,3α-diol-11,17-dione			0.10			0.11	
17	5β-Androstane-3α,6α-diol-17-one	9		0.15	0.47		0.19	0.67
18	5β-Androstane-3α,6α-diol-11,17-dione			0.06			0.05	
19	5β-Pregnane-1β,3α,17α,20β,21-pentol	10	3	0.29	0.46			
20	5β-Pregnane-1β,3α,17α,20β,21-pentol-11-one			0.12				
21	5β-Pregnane-3α,17α,20β,21-tetrol-1-one	11		0.59	0.70			
22	5β-Pregnane-3α,17α,20β,21-tetrol-1,11-dione			0.22				

in a total of five systems. Examination of these data show that the ΔR_{Mg} values for pairs 2 and 4 are about twice as large as those for pairs 1,3 and 5 (systems 1 and 4), that the values for pair 7 are nearly twice those for pairs 6, 8 and 9 (systems 2 and 5), and that the value for pair 11 is similarly greater than that of pair 10 (system 3). When these results are related to the indicated structures of the steroids, it is evident that this marked enhancement of the effect of the C-11 carbonyl group is manifest only in those compounds (4, 8, 14 and 22) which also bear a carbonyl group at C-1.

This effect of the 1,11-diketo system on mobility also was noted in the case of certain steroidal enol methyl ethers which were described in an earlier publication[2]. Structure assignments were made from their NMR spectra and there was a good correlation between the proposed structures and the observed constants. But as Table III illustrates, the relative mobilities, in system 6, of the 11-keto ethers (compound 24, mobile and compound 26, polar) are the reverse of those noted for the corresponding 11-deoxy ethers (compound 23, polar and compound 25, mobile). The basis

TABLE III

CONTRIBUTION OF THE C-11 CARBONYL GROUP IN STEROIDAL ENOL METHYL ETHERS

Compound No.	*Compound*	*Pair*	*System*	R_F	ΔR_{Mg}	*System*	R_F	ΔR_{Mg}
23	5β-Androst-1-ene-1-methoxy-3,17-dione	12	6	0.55	0.81	7	0.23	0.31
24	5β-Androst-1-ene-1-methoxy-3,11,17-trione			0.16			0.13	
25	5β-Androst-2-ene-3-methoxy-1,17-dione	13		0.70	1.30		0.42	0.33
26	5β-Androst-2-ene-3-methoxy-1,11,17-trione			0.10			0.25	

for this discrepancy became apparent when the mobilities of all four ethers were determined using adsorption (thin-layer, silica gel) chromatography. It will be noted (Table III, system 7) that the structure–mobility relationship is here a consistent one. TAMM[13] also noted, in the case of the chromatography on alumina of the enol methyl ethers derived from 5α-cholestane-1,3-dione, that the 3-methoxy derivative was the mobile member.

One of our objectives in the earlier and present study was to prepare pairs of epimeric 1-ols and to determine their relative mobilities in partitioning systems. Four such pairs, chiefly 17-ketosteroids and all in the 11-deoxy series, have been prepared to date. Their structures and R_F values in a total of six systems are given in Table IV. Each of the pairs of 17-ketones were chromatographed in four systems of widely

TABLE IV

R_F VALUES OF STEROIDAL EPIMERIC 1-OLS[a]

Compound No.	*Compound*	*Pair*	*System*	R_F	*System*	R_F	*System*	R_F	*System*	R_F
27	5β-Androstane-1α,3α-diol-17-one (e)	14	8	0.16	9	0.22	10	0.27	11	0.14
15	5β-Androstane-1β,3α-diol-17-one (a)			0.17		0.21		0.23		0.12
28	5α-Androstane-1α,3β-diol-17-one (a)	15		0.17		0.25		0.27		0.16
29	5α-Androstane-1β,3β-diol-17-one (e)			0.16		0.22		0.25		0.14
30	Androst-5-ene-1α,3β-diol-17-one (a)	16		0.12		0.13		0.13		0.10
31	Androst-5-ene-1β,3β-diol-17-one (e)			0.13		0.20		0.22		0.13
32	5β-Pregnane-1α,3α,17α,20β,21-pentol (e)	17	12	0.26	13	0.26				
19	5β-Pregnane-1β,3α,17α,20β,21-pentol (a)			0.23		0.24				

[a] The axial (a) or equatorial (e) configuration of the hydroxyl group at C-1 is indicated in parenthesis after the name of each compound.

varying composition, and the pregnane pair in two other, still different, systems. It is to be remarked that although the R_F differences in most cases are very small, such differences (as opposed to R_F values) could be demonstrated repeatedly.

In the case of the 5β-androstanes (pair 14), that member bearing an equatorial hydroxyl group at C-1 was the more mobile in three systems out of four. The greater mobility of the equatorial member also was evident with the 5β-pregnanes (pair 17) and with the androst-5-enes (pair 16). However, the reverse relationship holds for the 5α-androstanes (pair 15), where the member bearing an axially-oriented hydroxyl group at C-1 was the more mobile in all systems*.

In Table V we have assessed, in terms of ΔR_{Mg} values, the contribution to polarity of hydroxyl groups introduced at C-1 and at other positions in a number of 5α- and 5β-androstanes and androstenes. Two systems of contrasting composition

TABLE V

CONTRIBUTION OF HYDROXYL GROUPS IN 5β-ANDROSTANES, 5α-ANDROSTANES, AND ANDROST-5-ENES

Compound No.	*Compound*[a]	R_F *values*[b] *in system*				ΔR_{Mg} *in system*	
		9		*10*		*9*	*10*
27	5β-Androstane-1α,3α-diol-17-one (e)	0.85	0.22	0.88	0.27	1.30	1.31
15	5β-Androstane-1β,3α-diol-17-one (a)	0.85	0.21	0.88	0.23	1.34	1.41
28	5α-Androstane-1α,3β-diol-17-one (a)	0.86	0.25	0.88	0.27	1.27	1.31
29	5α-Androstane-1β,3β-diol-17-one (e)	0.86	0.22	0.88	0.25	1.35	1.36
30	Androst-5-ene-1α,3β-diol-17-one (a)	0.84	0.13	0.88	0.13	1.55	1.70
31	Androst-5-ene-1β,3β-diol-17-one (e)	0.84	0.20	0.88	0.22	1.33	1.43
33	5β-Androstane-3α,6α-diol-17-one (e)	0.88	0.12	0.88	0.07	1.74	1.92
34	5β-Androstane-3α,7β-diol-17-one (e)	0.88	0.14	0.88	0.15	1.65	1.62
35	5β-Androstane-3α,11α-diol-17-one (e)	0.88	0.33	0.88	0.27	1.17	1.31
36	5β-Androstane-3α,11β-diol-17-one (a)	0.88	0.51	0.88	0.55	0.85	0.78
37	5β-Androstane-3α,15α-diol-17-one (e')[c]	0.88	0.31	0.88	0.29	1.22	1.26

[a] The configuration of the hydroxyl group being examined is indicated in parenthesis after the name of the compound.

[b] In each case, values in the left column are derived from the appropriate stem compounds (5β-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one, or androst-5-en-3β-ol-17-one), and those in the right column from the substituted stem compounds.

[c] Pseudoequatorial.

were used together with the appropriate 17-ketosteroids as compounds of reference.

These values are similar to those which we published earlier for a series derived from deoxycorticosterone[5] and, with the exception of those obtained for compound 36, are within the range for what may be termed "relatively unhindered" hydroxyl groups. We attach no particular significance to the small differences in ΔR_{Mg} values given by the two systems. As far as the 1-hydroxylated compounds are concerned, these results confirm those described in Table IV; in making these comparisons it should be recalled that R_F and ΔR_{Mg} values are inversely related.

* Recently we determined the relative mobilities of the epimeric 5α-androstan-1-ols[17] using a reversed-phase technique (system 14), and observed the same result: the axial member moved 104 mm from the origin in a running time of 24 h while its epimer moved 89 mm within the same time period.

DISCUSSION

Prior to considering explanations for the unusual polarity of the five 1,11-diketones (compounds 4, 8, 14, 22 and 26 in Tables II and III), it seems of interest to indicate other normal and abnormal properties of this class. JONES AND DIGIORGIO[14] have examined Dreiding models of compounds 4 and 8 (Table II and Fig. 1), and have determined their infrared spectra in chloroform and carbon disulfide solution. They reported that there was no more interaction between the C-1 and C-11 carbonyl groups

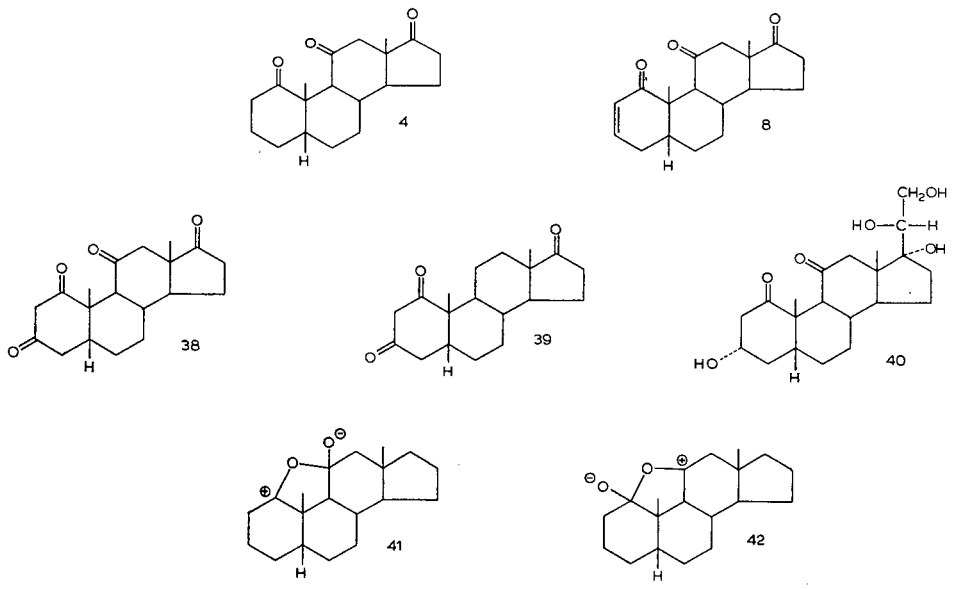

Fig. 1.

of these compounds, as judged by displacement of the C-11 band, than in 11,17-diketones generally. This is surprising since they noted that the two carbonyl groups of compound 4 are separated by only 2.8 Å (ring B chair conformation) while those of compound 8 are separated by 3.7 Å (ring B boat conformation). Since there was no marked absorption above 3000 cm^{-1}, it was concluded that neither steroid was appreciably enolized. In contrast, we have made two observations in this series which suggest the occurrence of marked physical interaction between the two carbonyl groups. The first is that the extinction coefficient, ε, in methanol of 5β-androstane-1,3,11,17-tetrone (compound 38 in Fig. 1) is only 6,400 or about one-half that of the corresponding 11-deoxy-β-diketone (compound 39, Fig. 1, ε = 12,650). The second observation is that 1,11-diketones, lacking carbonyl groups elsewhere, such as 3α,17α,20β,21-tetrahydroxy-5β-pregnane-1,11-dione (compound 40, Fig. 1), are very resistant to catalytic or metal hydride reduction, whereas the corresponding 11-deoxy-1-ketone readily is reduced to the equatorial alcohol. Both of these observations are discussed in detail in the earlier paper[2].

We believe that the observed hyperpolarity of 1,11-diketones in partitioning

systems is a consequence of the proximity of the carbonyl groups. The effect of their close approach, as 1,4-diones in a *cisoid* relationship, is to depolarize both carbonyl groups and to reduce the positive charge on each carbon atom. It seems likely that the associated repulsive forces are sufficient to distort the conformation of the molecule with a consequent change in its distribution characteristics. In addition, the carbonyl group area could serve as a site for association with components of the stationary phase, or for the generation of an ionized species; both would serve to increase the solubility of the compound in the immobile phase and thus provide the observed result. Professor D. H. R. BARTON, in a personal communication, has suggested that the abnormal polarity of *cisoid* 1,4-diones might be explained by supposing an equilibrium between the classic structure and such polar structures as compounds 41 and 42 in Fig. 1. The infrared evidence against enolization does not prevent its occurrence under the conditions prevailing in chromatography, but it is to be remarked that these diketones do not behave chromatographically like enols. They do not streak in neutral systems and the capacity of such systems seems little altered on adding acetic acid. The observation that the mobility–structure relationship for the enol methyl ethers (Table III) is an irregular one in partitioning systems but regular on adsorption chromatography is in accord with the view[15] that the conformation adopted by a molecule adsorbed on a solid surface is not necessarily its preferred conformation in solution.

In considering the data of Tables IV and V, it is apparent from the small differences in R_F that the contribution to polarity of each epimer is very similar. In the 5β-androstane series, that member bearing an axially-oriented hydroxyl group at C-1 (compound 15) is less mobile in three systems out of four, thus providing an exception to the rule[16] which states that the axial member of an epimeric pair is the more mobile. Examination of a Dreiding model of compound 27 in the all-chair conformation (Fig. 2)

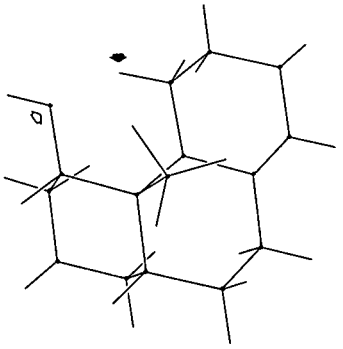

Fig. 2. Dreiding model of rings A (left), B and C of 5β-androstan-1α-ol, photographed from the front (β) face. Open arrow indicates oxygen atom of the equatorial (α) hydroxyl group at C-1; solid arrow points to the equatorial (α) hydrogen atom at C-11.

appears to offer an explanation: the distance between the oxygen atom of the equatorial hydroxyl group at C-1 and the 11α-hydrogen atom is very small, of the order of 1.6 Å. The resulting interaction would serve to reduce the activity of the hydroxyl group, presumably by limiting its association with components of the stationary phase.

The relative mobilities of the members of the 5β-pregnane pair (compounds 19

and 32) and of the androst-5-ene pair (compounds 30 and 31) also are anomalous and apparently for the same reason, namely the close approach, in compounds 31 and 32, of the equatorial hydroxyl group at C-1 and the 11α-hydrogen atom.

But the results obtained in the 5α-androstane series cannot be explained in these terms. Here, in accordance with the rule[16], the axial member (compound 28) is the more mobile even though a Dreiding display of its epimer (compound 29) shows that the equatorial hydroxyl group at C-1 closely approaches the 11α-hydrogen atom.

It is possible that conformational distortion, induced by the proximity of the oxygen function at C-1 and the 11α-hydrogen atom, may account for the observed differences in chromatographic behavior in, for example, the 5α-androstane and androst-5-ene pairs. While this factor may operate in all four cases, it might be supposed that it would have unequal effects in the 5α-androstane and androst-5-ene series because the double bond in the latter tends both to flatten rings A and B and to limit conformational mobility. It might be added that in those epimers not involved in C-1 : C-11 interaction, namely compounds 15, 19, 28 and 30, the axially-oriented hydroxyl group at C-1 appears to be relatively unhindered, sharing simple 1–3 interactions in all cases.

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Short communications

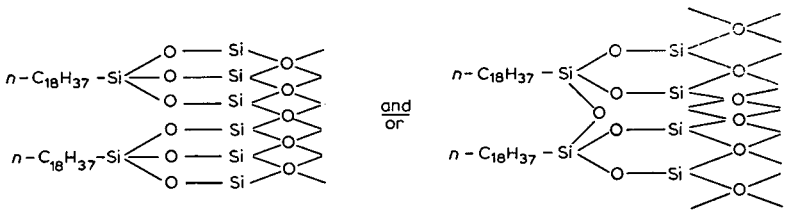
CHROM. 3716

A new approach to liquid partition chromatography

Liquid adsorption chromatography, in columns and on thin layers, is widely used in the separation and analysis of lipophilic organic mixtures. In general it provides separations by compound type and, in some instances, suffers the disadvantage that polar or labile substances cannot be recovered completely, or unchanged, from the adsorbent. Partition systems offer the possibility of separation within homologous series and, because less chemisorbtive or "active" materials are used than in adsorption chromatography, are much less likely to hinder complete recovery of unchanged, separated components. Hence there is an interest in developing practically useful liquid partition chromatographic systems.

That rather little work in this field has been described is largely due to the problem of finding suitably immiscible phase pairs. If the "stationary" phase is even slightly miscible with the moving phase rather elaborate experimental precautions are necessary to maintain the column phase distribution constant. Where truly immiscible phase pairs are used, it is not surprising that separations are often poor. This is because immiscible phase pairs differ very considerably in polarity (*e.g.* water–hexane) and sample components tend to have partition coefficients in such a system tending either to zero or infinity.

We have adopted a procedure described by ABEL, POLLARD, UDEN AND NICKLESS[1] for overcoming column "bleed" in gas–liquid chromatography and successfully applied it to overcome the problem discussed above in liquid–liquid chromatography (LLC). In this procedure a porous solid, the surface of which is covered with hydroxyl groups, is reacted with an organo-substituted silyl halide. The product of the condensation reaction is a particle which remains porous and is covered with a "fur" of organic matter of the type[1]:



In order to assess the potential of these "anchored" stationary phases in LLC we prepared "octadecyl kieselguhr" by reacting octadecyl silyl chloride (Aldrich Chemical Co., Ann Arbor, U.S.A.) with kieselguhr (100–120 mesh, for gas chromatography) according to POLLARD *et al.*'s procedure. The resulting stationary phase consists essentially of a skin of alkyl groups on the kieselguhr which, in conjunction with hydrocarbon mobile phases, should provide a much more practically useful range of solute partition since the phases differ little in polarity. The material we prepared was found by thermogravimetric analysis to contain 20 weight percent of organic

matter. Prolonged extraction or washing of this material with organic solvents failed to remove any organic matter from the stationary phase. Simple test dye mixtures were separated on short columns packed with the anchored phase and further, more sophisticated chromatography with columns of octadecyl kieselguhr is in hand. By the use of differently substituted silanes a wide range of polarity for the stationary phase is accessible; use of other solids such as silica gel or alumina will also modify the polarity of anchored phases.

It appears to us that the family of anchored phases proposed above offers the best (if not the only) present possibility for LLC of lipophilic mixtures without the necessity for precision thermostatting and careful pre-equilibration of conventional "immiscible" phase pairs.

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1 F. W. ABEL, F. H. POLLARD, P. C. UDEN AND G. NICKLESS, *J. Chromatog.*, 22 (1966) 23.

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CHROM. 3695

TAS, ein Thermomikro-Abtrenn- und Applikationsverfahren gekoppelt mit der Dünnschicht-Chromatographie

Viele organische und anorganische Substanzen sind bei höherer Temperatur flüchtig. Sie lassen sich auf diesem Wege einfach und schnell von nichtflüchtigen Stoffen abtrennen. Beim TAS-Verfahren bringt man die zu untersuchende Probe in eine Glaspatrone, deren eines Ende zu einer Kapillare verjüngt ist. Die gefüllte Patrone wird dann in den bereits auf eine bestimmte Temperatur erhitzten TAS-Ofen (Fig. 1 und 2) eingeschoben. Die flüchtigen Substanzen gelangen nun direkt auf die

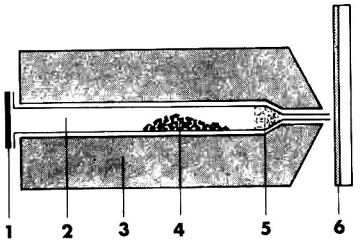

Fig. 1. Schemazeichnung des TAS-Ofens im Längsschnitt mit Patrone und DC-Platte. 1 = Abdichtung; 2 = Glaspatrone; 3 = Heizblock (Ofen); 4 = Probe; 5 = Glaswolle; 6 = DC-Schicht.

davor gehalterte DC-Platte. Der Aufdampfvorgang beträgt je nach Substanz nur 15–90 Sek. und die Startpunkte sind scharf ausgebildet.

Man umgeht auf diese Weise eine zeitraubende Herauslösung der Substanzen durch Extraktion mit Lösungsmitteln und anschliessendem Einengen und Auftragen mit Pipette. Die Thermomikro-Abtrennung lässt sich auch stufenweise oder kontinuierlich bei verschieden hoher Temperatur durchführen. Die DC-Platte wird dabei schrittweise in einer Richtung verschoben. Man kann so eine weitere Vorfraktionierung erreichen.

Das TAS-Verfahren eröffnet zahlreiche weitere Möglichkeiten. Einige sind in der Fig. 3 schematisch zusammengestellt. So kann z.B. durch eine Zusatzfüllung der Patrone mit Kieselgel von bestimmtem Wassergehalt eine Art "Ultramikrodestillation" durchgeführt werden (Fig. 3a). Auch eine "Heissdampfextraktion" ist durch Injektion von einigen Mikrolitern Lösungsmittel möglich (Fig. 3b und 3c).

Ferner ist das Austreiben in Kombination mit einem Inertgasstrom möglich (Fig. 3d).

Die in den Fig. 3e–3h zusammengestellten Techniken sind für jeden, der sich mit Synthese oder Abbau chemischer Substanzen beschäftigt, von Interesse. Auf diesem Wege lassen sich thermische Umlagerungen, Dehydratisierungen, Dehydrierungen, Alkalischmelzen, Zinkstaubdestillationen usw. als Vorversuch durchführen, und das Ergebnis lässt sich direkt anschliessend mittels der DC erkennen. Beachtenswert ist auch die Möglichkeit des direkten Aufdampfens von Substanzen auf Quarzscheiben oder KBr-Presslinge mit dem Ziel der spektroanalytischen Unter-

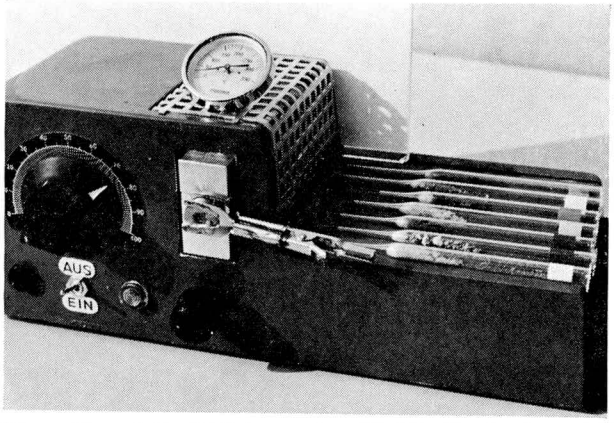

Fig. 2. Komplette TAS-Apparatur, Modell 68 mit eingeschobener Patrone; rechts daneben die nachfolgenden bereits gefüllten Patronen (Fa. Desaga, Heidelberg, D.B.R.).

suchung. Auf normalen Glasplatten im Objektträgerformat lassen sich kristalloptische Identifizierungen ebenso durchführen wie Tüpfelreaktionen usw. Der TAS-Ofen ermöglicht ausserdem die Mikrokugelrohrdestillation bzw. Sublimation im Vakuum zur Schnellreinigung kleiner Substanzproben (Fig. 3i).

Eine weitere Anwendung des TAS-Verfahrens ist die gezielte Niedertemperatur-Pyrolyse (150–450°) im Mikromassstab in direkter Kopplung mit der DC. Sie kann ebenfalls zur Substanzidentifizierung herangezogen werden. So erhält man z.B. beim TAS-Verfahren von Zuckern und Polysacchariden bei 275° und Erhitzungszeiten

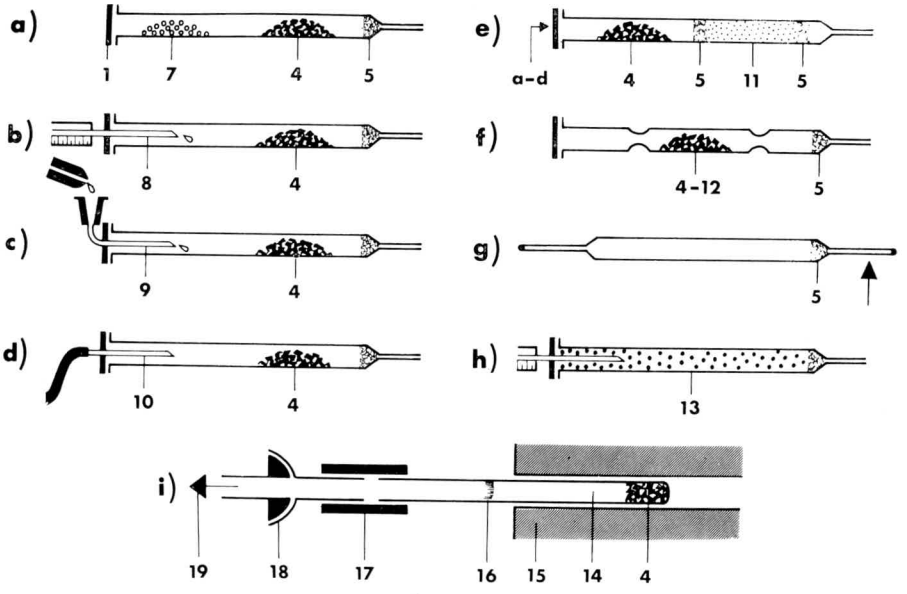

Fig. 3. Die verschiedenen Möglichkeiten der Beschickung und Ausführung der Patronen sowie einige weitere Techniken (Einzelheiten s. Text und ref. 3; Ziffern 1–6 s. Fig. 1).

zwischen 30 und 120 Sek., sowie Einwaagen von 10 mg auf dem Chromatogramm Zonen von Furfural, Hydroximethylfurfural und Furfurylalkohol. Sind Lignine zugegen, wie z.B. bei Holz, Kleie oder Zeitungspapier, so treten nach der Sichtbarmachung zusätzliche Farbzonen beispielsweise vom Coniferylalkohol hervor. Auch bei anderen natürlichen und synthetischen Polymeren ist dieses Verfahren nützlich.

Gerätebeschreibung

Das Kernstück ist der elektrisch aufheizbare Aluminiumblockofen (Fig. 1 und 2) mit einer zentralen Bohrung zur Aufnahme der Patrone und einem Metallrundthermometer. Mittels eines Energiereglers lässt sich die gewünschte Ofentemperatur einstellen. Der Ofen ist in seiner Längsachse über einen Spindeltrieb (Drehknopf unter dem Ofen) um einige Millimeter beweglich. Auf diese Weise lässt sich der Abstand zwischen der Patronenspitze und der Oberfläche der DC-Platte auf den günstigen

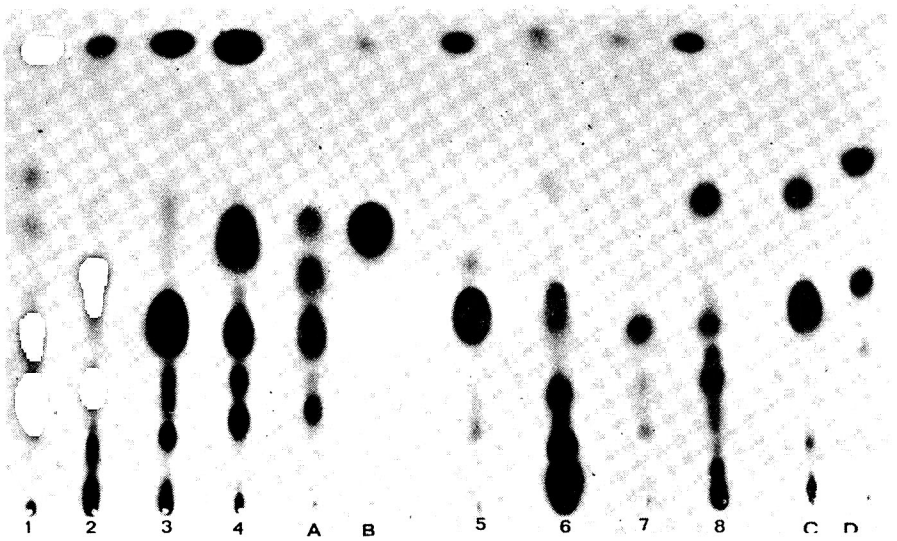

Fig. 4. Dünnschicht-Chromatogramm der flüchtigen Inhaltsstoffe von Labiatenblättern und -blüten nach Direktauftragung mit dem TAS-Verfahren. 1 = Folia Menthae; 2 = Herba Thymi; 3 = Folia Salviae, spanisch; 4 = Folia Salviae, dalmatinisch; 5 = Folia Menthae crispae; 6 = Folia Melissae; 7 = Folia Rosmarini; 8 = Flores Lavandulae. Testlösungen: A = Menthon (hR_F 60), Thymol (hR_F 50), Cineol (hR_F 35), Menthol + Campher (hR_F 20) B = Thujon; C = Linalylacetat (hR_F 60–65), Cineol (hR_F 35–40), Citral (hR_F 35–40); D = Buttergelb (hR_F 65–70), Sudanrot G (hR_F 40–45), Indophenol (hR_F 30).

Abstand von ca. 1 mm einstellen. Ein Projektionsscheinwerfer erleichtert diesen Einstellvorgang wesentlich. An der Rückseite des Gerätes ist eine Führungsschiene und ein Haltearm mit verstellbarer U-Schiene für verschieden grosse Glasplatten vorgesehen. Bei den TAS-Patronen handelt es sich um eine genau dimensionierte Spezialanfertigung aus Geräteglas 50. Der HD-Clip ermöglicht es, die TAS-Patrone mit einer Hand zu verschliessen und auch einen Wechsel bei heisser Patrone. Er garantiert einen dichten Verschluss und ermöglicht das Einstecken von Metallkapillaren, um Gase oder Lösungsmittel in die heisse Patrone einzuführen.

Die Zubehörteile, wie TAS-Patrone, HD-Clip (= Halte- und Dichtungsklammer) und Silikon-Dichtungsscheibchen sind in der Geräteschublade untergebracht. Eine gerillte Ablagefläche macht einen schnellen Wechsel der u.U. heissen Patronen möglich.

Handhabungsbeispiele

(a) Vorversuch. Einige Milligramm des unbekannten Gemisches in ein im Durchmesser genau in die Bohrung des TAS-Ofens passendes Reagenzgläschen füllen und einige Zentimeter tief einstecken. Heizung einschalten und feststellen, in welchem Temperaturbereich sich im kalten Teil des Reagenzgläschens ein Kondensat- oder Sublimatring bildet.

(b) Hauptversuch. Substanz in die TAS-Patrone füllen, verschliessen und in den aufgeheizten TAS-Ofen (Temperatur siehe Vorversuch) stecken. Abstand zur DC-Platte auf 1 mm regulieren. Nach der ebenfalls in einem Vorversuch ermittelten Aufdampfzeit (ca. 1 Min.) Patrone herausnehmen und DC-Platte um 1–2 cm verschieben.

(c) Reihenversuch. Zunächst alle zu untersuchenden Proben in verschieden markierte Patronen füllen und dann nacheinander die Direktaufgabe nach dem TAS-Verfahren durchführen. Auf eine 20 × 20 cm DC-Platte bringt man etwa acht bis zehn Proben und zwei Vergleichsgemische auf.

Anwendungsmöglichkeiten

Im Bereich der Analytischen Phytochemie wurden an nahezu 30 Drogen mit ätherischen Ölen die Vorteile dieses neuen Verfahrens demonstriert [1,3]. Aber auch manche Alkaloide, Amine, viele Cumarine, Anthrachinone, Purine, Rauschgifte lassen sich auf diesem Weg direkt der Dünnschicht-Chromatographie zuführen[2]. Das TAS-Verfahren wird, wie orientierende Versuche zeigten, auch auf folgenden Gebieten eine wertvolle Hilfe bringen: Rückstandsanalyse von Nahrungsmitteln und Gebrauchsgegenständen, ferner bei der Untersuchung von Kunststoffen und Weichmachern, beim Abtrennen von Holzschutzmitteln und Isolierungsmaterial. Auch die Abtrennung flüchtiger Elemente und Verbindungen aus Mineralproben und aus künstlichen anorganischen Gemischen ist auf diesem Wege möglich. Wertvoll ist das TAS-Verfahren ausserdem zur Reinigung kleiner Substanzmengen und für gezielte Umsetzungen im Mikromasstab.[3]

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Notes

CHROM. 3647

The anomerization of D-glucose as determined by gas chromatography

The mutarotation of D-glucose in various solvents has been studied extensively by polarimetric procedures[1–7]. More recently, other instruments were used to investigate this phenomenon. Nuclear magnetic resonance (NMR) was used in the study of glycopyranoses by NOBUO MORI *et al.*[8] and by RAO AND FOSTER[9]. PARKER[10] has investigated the behavior of carbohydrates in water by infrared spectroscopy.

SWEELY *et al.*[11] studied carbohydrates as their trimethylsilyl ethers by means of gas chromatography. Although since then many aspects of carbohydrate chemistry have been investigated by this method, the use of gas chromatography in the study of D-glucose anomerization has not been reported. In this investigation gas chromatographic procedures have been applied in the study of D-glucose anomerization in various solvents.

Experimental

A Perkin-Elmer 800 gas chromatography unit with a flame ionization detector was used in this work. The column was stainless steel, 6 ft. long by $^1/_8$ in. O.D. The coating was 8% neopentyl glycol adipate (NPGA) on silanized Chromosorb W. The column temperature was 140° programmed to 180° at 4° per min. Injection block temperature was 230°. The nitrogen flow rate was 32 ml per min and the stream was split 80–20 in favor of the atmosphere.

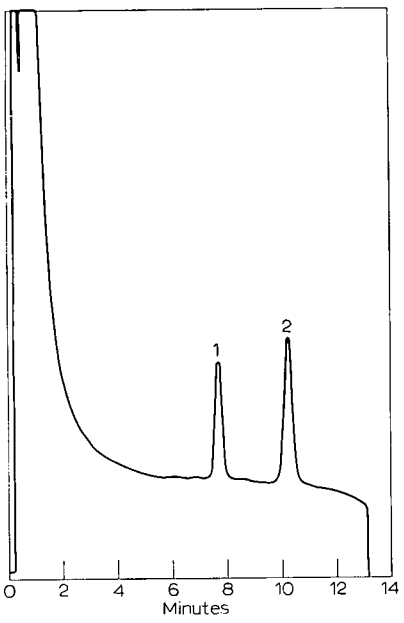

Fig. 1. Chromatographic determination of D-glucose anomers. 1 = α-D-glucose; 2 = β-D-glucose.

TABLE I

THE ANOMERIZATION OF α-D-GLUCOSE IN WATER, DEUTERIUM OXIDE, AND AQUEOUS BUFFERS

Time (min)	*Water*		*D_2O*		*pH 3.7*		*pH 7.1*		*pH 9.0*	
	% *alpha*	% *beta*	% *alpha*	% *beta*	% *alpha*	% *beta*	% *alpha*	% *beta*	% *alpha*	% *beta*
1	90.5	9.5	95.9	4.1	95.8	4.2	92.5	7.5	94.1	5.9
15	71.0	29.0	82.0	18.0	79.5	20.5	75.0	25.0	76.0	24.0
30	60.0	40.0	76.5	23.5	60.0	40.0	64.0	36.0	68.0	32.0
45	52.5	47.5	69.5	30.5	—	—	—	—	—	—
60	49.0	51.0	67.0	33.0	51.5	49.5	49.5	50.5	59.0	41.0
75			63.0	37.0	48.5	51.5	—	—	53.0	47.0
90			60.0	40.0	47.0	53.0	46.0	54.0	50.0	50.0
105			58.5	41.5	44.5	—	—	—	47.5	52.5
120			55.5	44.5	—	—	—	—	45.0	55.0
135			54.0	46.0	40.0	60.0	—	—	—	—
150			52.0	48.0	—		44.0	56.0	46.0	54.0
180			—	—	41.0	59.0	—	—	42.0	58.0
240			—	—			45.0	55.0		
20 h			41.0	59.0						
24 h	42.0	58.0								

Concentrated D-glucose solutions were made up in the various solvents and kept for the indicated time intervals. Aliquots of these solutions were then mixed with Tri-sil reagent (Pierce Chemical Co., Ill.) left at room temperature for 3 min and the trimethylsilyl ethers formed examined gas chromatographically. A Honeywell 1 mV single pen recorder was used. Peak height was taken as a measure of anomer concentration in the examined sample. A typical chromatogram is shown in Fig. 1.

The non-aqueous solvents were examined for water content prior to use in D-glucose anomerization studies. A Beckman GC-5 with a thermal conductivity detector was used. The column support was Porapak-Q and the column temperature was 130°. All other conditions were as recommended by Waters Associates, Inc., Framingham, Mass., manufacturers of Porapak-Q.

TABLE II

THE ANOMERIZATION OF β-D-GLUCOSE IN WATER, DEUTERIUM OXIDE, AND pH 9.0 BUFFER

Time (min)	*Water*		*D_2O*		*pH 9.0*	
	% *alpha*	% *beta*	% *alpha*	% *beta*	% *alpha*	% *beta*
1	14.0	86.0	3.5	96.5	15.0	85.0
15	22.0	78.0	17.0	83.0	24.0	76.0
30	27.0	73.0	24.0	76.0	32.0	68.0
45	32.0	68.0	29.5	70.5	—	—
60	37.0	63.0	32.5	67.5	39.0	61.0
75	39.0	61.0	—	—	—	—
90	41.0	59.0	35.0	65.0	44.0	56.0
105	40.5	59.5	—	—	—	—
120	42.0	58.0	37.0	63.0	41.0	59.0
180	40.0	60.0	—	—	—	—
210	—	—	—	—	45.0	55.0
1320	—	—	—	—	43.0	57.0
3 days	—	—	40.5	59.5	—	—

Results and discussion

The ratio of D-glucose anomers in water, deuterium oxide, and in aqueous buffers at various time intervals, is shown in Table I. The figures in Table I show that the anomerization of α-D-glucose proceeds at a faster rate in water than in deuterium oxide. The hydrogen ion concentration seems to have no effect upon the anomerization rate. D-glucose in water, deuterium oxide, and pH 9.0 buffer was also investigated with

TABLE III

ANOMERIZATION OF α-D-GLUCOSE IN FORMAMIDE AND DIMETHYLFORMAMIDE

Time (min)	*Formamide*		*Dimethylformamide*	
	% alpha	*% beta*	*% alpha*	*% beta*
1	96.7	3.3	98.7	1.3
15	82.0	18.0	98.3	1.7
30	68.0	32.0	98.6	1.4
45	59.0	41.0	—	—
60	55.0	45.0	—	—
150	46.0	54.0	—	—
210	—	—	97.6	2.4
245	44.0	56.0	—	—

β-D-glucose as the starting material. The results obtained are shown in Table II. A comparison of the figures in Tables I and II shows that the same equilibrium is reached whether the starting material is α- or β-D-glucose. However, the rate of anomerization of the α form to the β form seems to be faster than the reverse process.

The non-aqueous solvents contained less than 0.03% water except for the

TABLE IV

ANOMERIZATION OF α-D-GLUCOSE IN PYRIDINE AND PYRIDINE–WATER MIXTURES

Time (min)	*100% pyridine*		*90% pyridine*		*80% pyridine*		*50% pyridine*	
	% alpha	*% beta*	*% alpha*	*% beta*	*% alpha*	*% beta*	*% alpha*	*% beta*
1	—	—	97.1	2.9	95.0	5.0	79.0	21.0
15	—	—	88.5	11.5	63.0	37.0	40.0	60.0
20	95.3	4.7	—	—	—	—	—	—
30	—	—	83.0	17.0	51.0	49.0	42.0	58.0
45	93.0	7.0	—	—	46.5	53.5	—	—
60	92.2	7.8	69.0	31.0	44.5	55.5	—	—
75	—	—	65.0	35.0	43.0	57.0	—	—
90	—	—	—	—	43.5	56.5	—	—
105	—	—	57.5	42.5	—	—	—	—
120	86.5	13.5	54.5	45.5	—	—	—	—
170	82.5	17.5	48.5	51.5	—	—	—	—
195	—	—	46.0	54.0	—	—	—	—
240	75.5	24.5	45.0	55.0	—	—	—	—
300	71.0	29.0	—	—	—	—	—	—
24 h	47.0	53.0	43.0	57.0	—	—	—	—
29 h	46.0	54.0	—	—	—	—	—	—
120 h	45.5	54.5	—	—	—	—	—	—

TABLE V

ANOMERIZATION OF α-D-GLUCOSE IN DIMETHYL SULFOXIDE (DMSO) AND DMSO–WATER MIXTURES

Time	*DMSO*		*80% DMSO*		*50% DMSO*	
	% alpa	*% beta*	*% alpa*	*% beta*	*% alpa*	*% beta*
1 min	98.3	1.7	98.7	1.3	—	—
15 min	98.3	1.7	97.3	2.7	94.0	6.0
30 min	96.2	3.8	97.2	2.8	92.3	7.7
60 min	96.5	3.5	—	—	—	—
105 min	—	—	—	—	88.0	12.0
135 min	—	—	96.7	3.3	—	—
150 min	—	—	—	—	80.0	20.0
270 min	—	—	—	—	45.0	55.0
300 min	—	—	95.7	4.3	—	—
16 h	96.2	3.8	—	—	—	—
24 h	—	—	—	—	47.0	53.0
84 h	—	—	77.0	23.0	—	—
100 h	95.6	4.4	—	—	—	—
105 h	—	—	67.0	33.0	—	—
225 h	—	—	53.0	47.0	—	—
300 h	—	—	52.0	48.0	—	—
336 h	92.0	8.0	—	—	—	—

formamide, which contained 0.17% water. The results obtained with formamide and dimethylformamide (DMF) are shown in Table III. The figures show that no anomerization occurred in the DMF while in the formamide there was anomerization and an equilibrium was reached within 3–4 h.

The behavior of α-D-glucose in pyridine and pyridine–water mixtures is shown in Table IV. The equilibrium between α and β anomers is established at faster rates in the mixtures containing larger amounts of water, however anomerization does occur even in the 100% pyridine.

In dimethyl sulfoxide (DMSO) the α-D-glucose remained unchanged even after 300 h. The results of this experiment and of tests conducted in DMSO–water mixtures

TABLE VI

ANOMERIZATION OF α-D-GLUCOSE IN 50% DIOXANE

Time (min)	*50% dioxane*	
	% alpha	*% beta*
1	100	—
15	94	6.0
30	90	10.0
60	83.5	16.5
90	77.8	22.2
120	73.2	26.8
150	70.0	30.0
180	66.0	34.0
210	62.2	37.8
240	58.0	42.0
270	59.6	40.4
22 h	45.0	55.0
46 h	44.0	56.0

are shown in Table V. It can be seen from the figures in the table that the anomerization of α-D-glucose proceeds very slowly in DMSO–water mixtures. The α-D-glucose anomerization in 50% pyridine is about 48 times faster than in 50% DMSO.

The behavior of α-D-glucose in 50% dioxane is shown in Table VI. The anomerization is again comparatively slow. It proceeds at a rate similar to the one found for 50% DMSO.

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CHROM. 3646

Gas-chromatographische Untersuchung von C-Acylmalonestern

Bei den zur Zeit am hiesigen Institut laufenden Untersuchungen an C-Acylmalonestern, über die noch später ausführlich berichtet werden wird, ist das Problem aufgetaucht, eine Methode zur Reinheitsprüfung der genannten Verbindungen zu finden.

Da alle gas-chromatographischen Versuche mit Stahlsäulen und oberflächenaktiven Trägermaterialien das gleiche Ergebnis, nämlich vollständige Zersetzung der im Titel genannten Ester, liefern, wird das Augenmerk auf Glassäulen und Glaskugeln als Trägermaterial gerichtet[1].

Herstellung der Trennsäule

Belegungen mit verschiedenen stationären Phasen zeigen, dass SE-52 für die Lösung des aufgetauchten Problems am besten geeignet ist. Dabei hat sich eine Vorbehandlung von Säule und Trägermaterial durch Waschen mit HCl und HF und anschliessendes Silanisieren mit Dimethyl-dichlorsilan als vorteilhaft erwiesen. Man spült dazu die leere Säule mit einer Mischung aus Toluol und 10% Silan und saugt anschliessend trocken. Beim Belegen der Glasskugeln werden der Lösung der stationären Phase ebenfalls 5% Silan zugesetzt.

Ist bereits eine in der üblichen Weise gefüllte Glassäule vorhanden, so kann sie durch mehrmaliges Einspritzen von Dimethyldichlorsilan nachbehandelt werden[2].

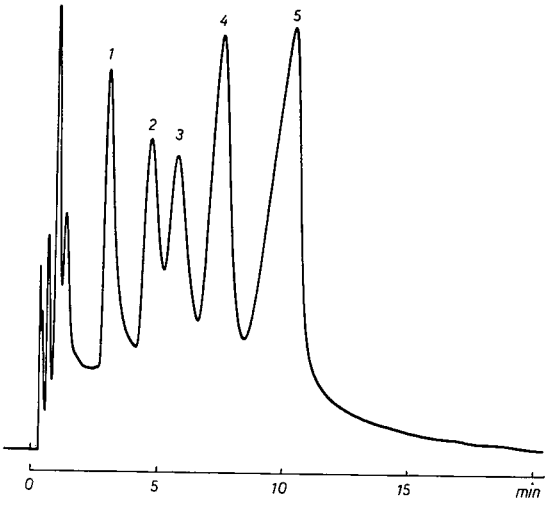

Fig. 1. Chromatogramm verschiedener C-Acylmalonester. Probenvolumen 0.7 μl; Ofentemperatur 125°; Glassäule 150 cm; Durchmesser 0.4 cm; Säulenfüllung 0.2% SE-52 auf Glaskugeln Durchmesser 0.19–0.20 mm; Säule und Trägermaterial behandelt mit HCl/HF und Dimethyldichlorsilan; Trägergas 29 ml/min N_2; FID Empfindlichkeit × 1000. Bezeichnung der Peaks: 1 = C-Acetylmalonester; 2 = C-Propionylmalonester; 3 = C-Isobutyrylmalonester; 4 = C-*n*-Butyrylmalonester; 5 = Enolacetat des C-Acetylmalonesters.

Anwendung

Die Zersetzung der Proben lässt sich auch mit diesen Säulen nicht restlos unterbinden, kann aber durch Variieren von Temperatur und Säulenlänge soweit reduziert werden, dass eine Identifizierung und Reinheitsprüfung der C-Acylmalonester erfolgreich durchführbar ist.

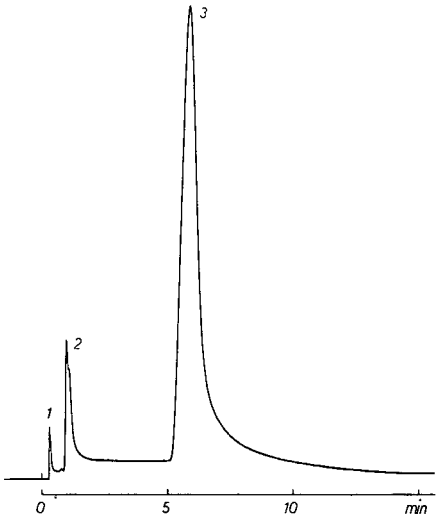

Fig. 2. Chromatogramm von C-Isobutyrylmalonester. Probenvolumen 0.1 μl; übrige Bedingungen wie bei Fig. 1. Bezeichnung der Peaks: 1 und 2 = Verunreinigungen; 3 = C-Isobutyrylmalonester.

Das Chromatogramm in Fig. 1 zeigt die Trennung eines Gemisches aus mehreren C-Acylmalonestern. Der letzte Peak entspricht dem Enolacetat des C-Acetylmalonesters, das bei der Darstellung des letztgenannten als Nebenprodukt erhalten wird. Die kurz nach der Front auftretenden Peaks stammen von Verunreinugungen, die bereits in den einzelnen Ausgangsprodukten der Mischung vorliegen.

Am Beispiel des Isobutyrylmalonesters (Fig. 2) sollen die Verhältnisse bei der Trennung genauer besprochen werden. Peak 1 und 2 zeigen die schon erwähnten Verunreinigungen an. Peak 3 ist dem unzersetzten Ester zuzuordnen. Der Nullinien-

TABELLE I

ZUSAMMENHANG ZWISCHEN ZERSETZUNGSGRAD UND VERWEILZEIT AM BEISPIEL DES C-PROPIONYLMALONESTERS BEI 125°

Verweilzeit (min)	*Unzersetzter Anteil (%)*
1.2	94.25
3.7	78.09
5.5	62.96
12.6	43.96

versatz zwischen Peak 2 und 3 wird durch einen Zersetzungsvorgang verursacht, dem alle untersuchten C-Acylmalonester während des Wanderns durch die Trennsäule unterliegen. Der Grad dieser Zersetzung hängt von Temperatur und Verweilzeit in der Trennsäule ab.

Zur Reinheitsprüfung verkürzt man daher die Säule—und damit die Verweilzeit—soweit, dass noch eine ausreichende Trennung von Verunreinigung und Hauptsubstanz gewährleistet ist (siehe Fig. 3a und b).

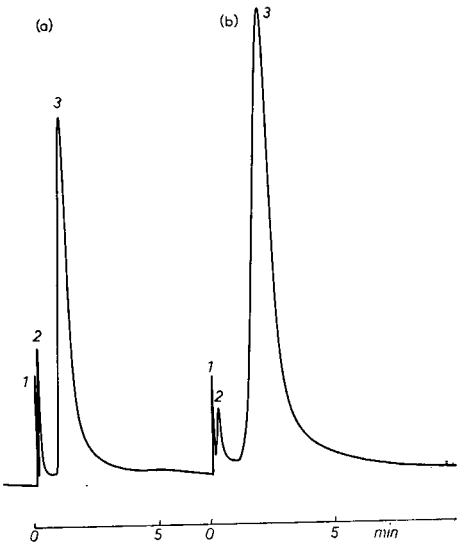

Fig. 3. Chromatogramm von C-Propionylmalonester (a) und C-*n*-Butyrylmalonester (b). Probenvolumen je 0.1 μl; Ofentemperatur 115°; Glassäule 30 cm; Durchmesser 0.3 cm; übrige Bedingungen wie bei Fig. 1. Bezeichnung der Peaks (a) und (b): 1 und 2 = Verunreinigungen; 3 = Substanz.

Apparatives

Da das zur Verfügung stehende Gerät nur über einen Metalleinspritzblock verfügt, war eine Lösung zu finden, die eine direkte Dosierung auf die Trennsäule ermöglicht. Dazu wird der Anfang einer Glassäule rechtwinkelig abgebogen und zwischen den Asbestdichtungen von Ofenraum und Ofenhaube hindurchgeführt. Fixieren der Ofenhaube in entsprechender Höhe ermöglicht den Einbau einer vom Ofenraum unabhängigen Heizung für die Einspritzzone.

Im Anschluss an die Glassäule müssen die aufgetrennten Komponenten die übliche Detektorzuleitung aus Stahl durchlaufen, wo sie sich infolge der hohen Temperatur zersetzen. Es ist anzunehmen, dass die in den Chromatogrammen auftretende Schwanzbildung auf solche Zersetzungsvorgänge zurückzuführen ist, die nach dem Durchlaufen der Trennsäule durch den Kontakt mit Metallteilen ausgelöst werden.

Die gas-chromatographischen Analysen sind mit einem Fraktometer F6/4 der Firma Perkin Elmer mit Flammenionisationsdetektor durchgeführt worden. Quantitative Messungen sind mit Hilfe eines elektronischen Integrators D 2 derselben Firma

erfolgt und die Chromatogramme über einen 2 mV Servogor Potentiometerschreiber der Firma Goerz Elektro aufgezeichnet worden.

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J. Chromatog., 37 (1968) 108–111

CHROM. 3675

A general screening method for urine constituents utilising gas–liquid chromatography

Few of the many constituents of urine are amenable to GLC without prior conversion to suitably volatile derivatives. For this purpose conversion to trimethylsilyl derivatives is often very suitable. Various silylation procedures have proved more or less successful according to the types of compound examined but a recent important advance has been achieved through the introduction of bis(trimethylsilyl) acetamide (BSA)[1,2]. This reagent rapidly and quantitatively silylates alcohols, enols, phenols, carboxylic acids, amines, amides, ureas, heterocyclic compounds such as purines and pyrimidines and has been shown to yield derivatives suitable for GLC with such complicated molecules as steroid glucuronides[3] and sugar phosphates[4]. It may thus be considered as a general reagent for GLC purposes.

Similarly, although a bewildering variety of liquid phases are still in use, interest seems gradually to be focussing on a few siloxane polymers as being most generally useful. These give excellent results with most trimethylsilyl derivatives and are not easily overloaded; relatively enormous quantities of urea, for instance, interfere only to the extent of obscuring immediately adjacent compounds on chromatograms.

It seemed to us that it should now be technically possible to develop a GLC method capable of screening urines for many types of compound in one operation. Such a method would have obvious practical limitations owing to overlaps and to wide quantitative differences between various compounds excreted, but should suffice to detect moderate pathological increases in relatively major urine constituents and larger increases in minor ones. Within these limitations such a general technique would be of obvious value in many pathological conditions and would doubtless further extend the range of diseases in which chemical analysis is of diagnostic value.

Experimental

Gas chromatography. The instrument used was a Pye 104 series dual-column model 64 with 1.5 m × 4 mm I.D. coiled glass columns. A hydrogen flame-ionisation

detector was used with a hydrogen flow-rate of 50 ml/min. The detectors were maintained at 280°. Injection heaters were used to give an initial temperature at the point of injection about 80° above the temperature at which programming (2°/min from 100° to 250°) was started. Argon at a flow rate of 50 ml/min was used as carrier gas.

Various columns were assessed roughly for performance in terms of the ratios of peak heights to base-widths and on the ease with which the excess reagent and solvent were eliminated to give a low, stable base-line. Columns investigated included QF-1 (3% on Gas Chrom Q, 85–100 mesh), SE-30 (10% on siliconised Celite, 100–120 mesh), polyphenylether (6 ring), XE-60, W-98, SE-52, OV-1 and OV-17 (all 10% on Diatoport S, 80–100 mesh). The column containing W-98 was selected as being of average performance and more likely to reveal potential technical difficulties at an early stage than would the better columns (SE-52, OV-1 and OV-17). W-98 (Hewlett-Packard Ltd., now listed as W-982) is described as a silicone gum rubber containing methyl and vinyl groups.

Chromatograms were recorded on a Leeds and Northrup Speedomax W recorder using a chart speed of 0.5 in./min. The ionisation amplifier attenuator was set at 1×10^3 to give full scale deflection at 10^{-9} A.

Preliminary experiments. All silylations were carried out with a 2:1 mixture of solvent and BSA. An ampoule of the reagent (Sigma; usually a very generous 1 ml) was opened, the contents mixed with 2 ml solvent and the mixture used at once.

Comparison of chromatograms carried out after varying amounts of evaporated urine (see below) had been treated with 0.5 ml of pyridine–BSA (2:1) indicated that, as the amount of reagent became increasingly insufficient to effect complete silylation, a few peaks disappeared from chromatograms at a stage when the majority were not noticeably affected. Among such peaks hippuric acid was recognised; the amide grouping in this compound is silylated with relative difficulty. Hence a suitable higher homologue, benzoyl-DL-phenylalanine (BPA), which is not naturally present in urine

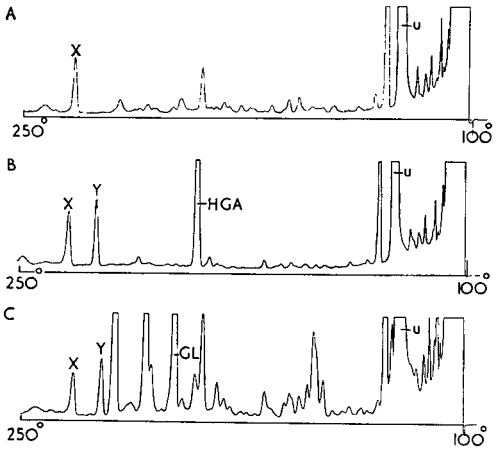

Fig. 1. Typical chromatograms of evaporated urine extracts. In each case extract equivalent to 2 μg urinary creatinine was chromatographed. Peaks U, X and Y represent urea, BPA standard and docosane standard, respectively. (A) Normal healthy male subject. (B) Case of alcaptonuria showing a large peak due to homogentisic acid (HGA). (C) Case of Lignac's disease with multiple abnormalities, known to include aminoaciduria and glucosuria. Peak GL corresponds to glucose.

and which appears on chromatograms at a point where no significant peaks normally appear, was subsequently added to urine extracts in order to indicate the efficiency of silylation. Docosane was added as a standard to correct for the intrinsic errors of GLC.

All chromatograms were dominated by a large early peak due to urea (Fig. 1). Peaks appearing before this included reagent peaks and probablv some derived from compounds of appreciable volatility which may be erratically lost in the evaporation process. When chromatograms were compared variations in these early peaks were ignored and only those emerging after urea were considered.

Aliquots of one normal urine were processed as described below, using as silylating reagent a 1:2 mixture of BSA and one of the following solvents: pyridine, ethyl acetate, triethylamine, hexamethyldisilazane, acetonitrile and dioxan. Almost identical chromatograms resulted, dioxan giving rise to most differences. The least amount of undissolved material was obtained when pyridine was used. Addition of trimethylchlorosilane (0.125 ml) to the extract obtained using pyridine did not alter the chromatographic pattern.

Chromatography of urines. Urine was adjusted to pH 1–2. An aliquot containing 0.25 mg creatinine was placed in a 100 ml round-bottom flask, diluted with 10 vols (or more if convenient) ethanol and evaporated to dryness *in vacuo* at not greater than 30°. Standards of BPA (250 μg) in ethyl acetate (0.5 ml) and docosane (200 μg) in ethanol (2 ml) were added, together with a few millilitres benzene, and evaporation was repeated. The dry residue was treated with BSA–pyridine (1:2, 0.5 ml) and the flask tightly stoppered with a polypropylene stopper. After a few minutes, with occasional rotation of the flask, the crust of solids disintegrated and a solution containing usually only a little undissolved material was obtained. The mixture was allowed to stand overnight before aliquots (4 μl) were chromatographed as described above; no attempt was made to remove solids. Occasionally crystals appeared in the mixture: these could be readily redissolved by gentle warming and appeared to be a useful indication of incomplete silylation.

Ten adult urines examined by the above procedure were found to be silylated satisfactorily as judged by the ratio of the peak heights of the BPA and docosane standards. When additional BSA (0.125 ml) was added to the extracts and chromatography was repeated, using 5 μl aliquots, chromatograms were virtually identical with the originals. Infant urines, containing relatively much more urea, provided a much more stringent test of conditions and in a high proportion (14 of 20) of those examined silylation proved to be incomplete before addition of extra BSA; usually the BPA peak was completely absent. It is probably not worth examining such urines without increasing the amount of BSA relative to that of urine.

Discussion

The work described was undertaken with two objectives in mind, to determine whether it was technically possible to obtain good chromatograms from urines subjected to the minimum treatment, and to investigate conditions necessary to ensure consistent silylation of reactive constituents.

No troubles were encountered in connection with the first objective and there remains considerable latitude for such variations in conditions as experience may indicate to be advisable. Evaporation of urine may be carried out in various ways,

with or without the addition of ethanol and samples may be buffered if this is thought to be desirable. The method described seemed to be quicker than possible alternatives and to offer conditions sufficiently mild to minimise possible decomposition or reaction of urine constituents. Nevertheless it has been noted, for instance, that phenylpyruvic acid does not yield the expected peak in phenylketonuria urine or when added to normal urine or to aqueous urea. Very good chromatograms were obtained using a column of average performance, suggesting that suitable columns should be immediately available in most laboratories equipped for GLC. However, attention should be drawn to the excellent results obtainable with OV-1 and OV-17, the thermal stabilities of which are such that they may be used at temperatures well in excess of 300° if desired.

Although BSA appears to be the best available silylating reagent, to be fully effective it must be present in excess and in this respect has two obvious defects. First, it is extremely sensitive to atmospheric moisture and must be handled appropriately. It did not seem advisable to risk the variable amounts of hydrolysis which might have occurred in extracts if manipulations such as centrifugation had been attempted and for this reason pyridine extracts containing relatively little solid were preferred to extracts obtained using solvents such as dioxan which may themselves be more readily dried. Secondly, the present high cost of BSA dictates its use in an economical manner. The experiments outlined above indicate conditions offering a good chance of complete silylation but it is hoped that the reagent will eventually become cheap enough to be used more liberally. But in any case it is desirable to include a standard to indicate the presence of excess BSA in a reaction mixture. In this connection BPA was found to possess suitable properties; it is of course theoretically possible for BPA to be just completely silylated whilst other less reactive compounds present in a mixture remain unreacted but no indication of this was observed in practice.

It was noted that BPA present in an adequately silylated urine extract always yielded a rather taller peak than did a similar quantity of the pure substance. In the latter case a small poorly shaped peak emerged from the column just before the major peak, suggesting silylation to be incomplete. It seems possible that other urinary constituents catalyse the reaction between BPA and BSA. In the steroid field it has been shown that reaction between sterically hindered hydroxyl groups and BSA may be catalysed by addition of hydrogen chloride or trimethylchlorosilane[3]. In GLC methods involving conversion of compounds to derivatives, allowance should be made for the possibility that a compound may behave in a different manner when pure than when in admixture with other compounds.

Under the conditions described above normal urines yielded chromatograms showing numerous, mostly rather small, peaks (Fig. 1A). It is not possible to define accurately the amount of substance necessary to give an 'abnormal' peak since this will depend on whether the compound emerges early or late, on the normal variation of the peak and of any overlapping peaks, and on the sensitivity of the detector towards the compound, as well as on the experience of the observer in examining chromatograms. However, it seems likely that most substances will be readily detectable if excretion is of the order of 200 μg per mg urinary creatinine (calculated if necessary as the trimethylsilyl derivative). Because of the complexity of urine we envisage the method primarily as a means of detecting abnormalities in the first place, prior to detailed investigation by other means, rather than as a research tool in its own right.

However, it seems likely that more specific applications may be possible through the use of selective detectors (*e.g.* electron capture or thermionic). The method can be adapted to form a very rapid screening procedure. Reaction of functional groups with BSA is rapid and limited largely by solubility of compounds[2]; hence preliminary chromatograms of extracts may be performed within a few minutes, particularly if the mixture is warmed. Given a urine of known creatinine content and a GLC apparatus ready for use, it is easily possible to screen a urine within 1 h if temperature programming at 4°/min is employed.

Extreme examples of pathological results obtained are illustrated. Fig. 1B shows a simple case in which enormous quantities of a single abnormal compound (homogentisic acid) were excreted. The method was here entirely adequate in itself, requiring only recognition of the peak and confirmation of its identity using alternative columns. In Fig. 1C numerous abnormal peaks were observed; clearly, detailed examination of a urine as complex as this would require more specific and sophisticated techniques. Nevertheless even in such cases much provisional diagnostic information may be immediately available on inspection of the initial chromatograms when major peaks, although numerous, form a pattern characteristic of a particular condition.

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CHROM. 3652

An artefact caused by the binding of protein to dextran gel

Some proteins are strongly bound to dextran gel (Sephadex) in environments of low ionic strength[1]. We have noted that this can lead to a serious error when gel filtration is used to assess the dispersity of a protein solute[2]. The error arises from the fact that aggregates of one protein but of different sizes are in general bound in different proportions. Some examples illustrate this and indicate a remedy.

Our current experiments are concerned with immunoglobulins and their peptide chains. Buffers of low ionic strength are used in order to keep the heavy chains monodisperse, and an assessment of dispersity is often carried out by filtration through Sephadex G-150. One such examination of human immunoglobulin G (Cohn Fraction II, Commonwealth Serum Laboratories, Melbourne, Australia) on a freshly packed column is illustrated by curve A in Fig. 1. The protein was eluted as a single symmetrical peak,

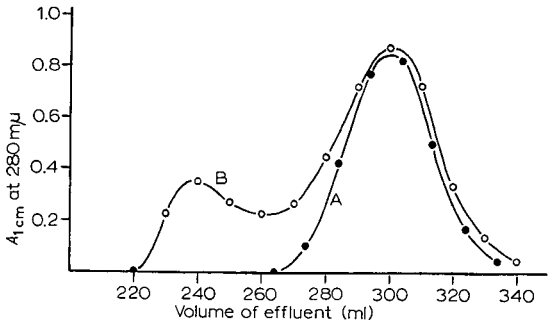

Fig. 1. Elution profiles given by human immunoglobulin G when two aliquots of one solution were passed successively through a column of Sephadex G-150, 3 × 90 cm, equilibrated with 4 m*M* sodium acetate buffer, pH 5.4. Curve A: first passage through a freshly packed column. Curve B: second passage, the column having been washed with 2 l of the acetate buffer between the runs. In each case 40 mg of protein was applied in 5 ml of the buffer.

suggesting that it was completely monodisperse. Such a finding, however, was at variance with ultracentrifugal analyses and examination of a second aliquot on the column (curve B, Fig. 1), both of which indicated the presence of about 20% aggregate. It is important to note that no change of buffer had intervened between the two runs in Fig. 1. The recovery of protein from the first run was 64%, from the second > 95%. Third and fourth runs, again with no intervening change of buffer, gave the same results as the second run.

This disparity between the first and subsequent runs through the column could be reproduced precisely after the column had been washed with 0.1 *M* NaOH, thereby eluting all the bound protein. The capacity for binding immunoglobulin G in the presence of the acetate buffer was approximately 0.03 mg protein per cm^3 of column volume. In accord with the results of GLAZER AND WELLNER[1] the binding was found to be irreversible as long as the buffer was not changed: exhaustive washing failed to elute any protein, and bound ^{14}C-labelled protein (partially reduced[3] and alkylated with ^{14}C-iodoacetamide) did not exchange with unlabelled protein put through

subsequently. It appears therefore that application of sufficient protein in the first run of a series will result in the binding sites being saturated and unavailable during the following runs.

Results similar to those in Fig. 1 were obtained when immunoglobulin heavy chains (40 mg) or hen ovalbumin (60 mg) were put through the same column in the same buffer. The capacity of the column for binding the latter was only about 0.01 mg per cm^3, but the disparity between the amounts of monomer and aggregate bound led to the aggregate being assessed as 11% of the total protein in the first run and 19% in subsequent runs.

Consider a protein present in solution in both monomeric and aggregated forms, not interconverting, passed through a column of dextran gel in which the former is incompletely and the latter completely excluded from the gel phase. The aggregate will move faster through the column, will be the first to encounter those binding sites accessible externally on the gel grains, and may saturate the sites so that they are unavailable to the monomer. Presumably the monomer will now encounter sites in the interior of the grains which were inaccessible to the aggregate. But there is clearly no guarantee that the proportions bound will be the same for each species. In the examples given the aggregate was always bound in greater proportion, but a reverse situation is conceivable.

The simplest way to avoid the error described is to put repeated aliquots through a single column, with no intervening change of buffer, until the elution profiles given in two successive runs are the same. We have found this procedure much more reliable than mere estimation of recoveries. In practice it need only imply a single priming application of protein to enable a column to be used repeatedly for assessing dispersity in different samples.

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Analysis of the pigments of black tea extracts by chromatography on acetylated Sephadex

Theaflavins, a term incorporating theaflavin itself and theaflavin gallates[1], are responsible for the desirable bright golden appearance of tea liquors[2] so that methods for measuring the amounts of these pigments are of value in assessing the quality of a tea. Methods which have been used for this determination are based on that of ROBERTS AND SMITH[3], in which the theaflavins are extracted from an aqueous infusion by isobutyl methyl ketone, purified to some extent by washing with sodium bicarbonate

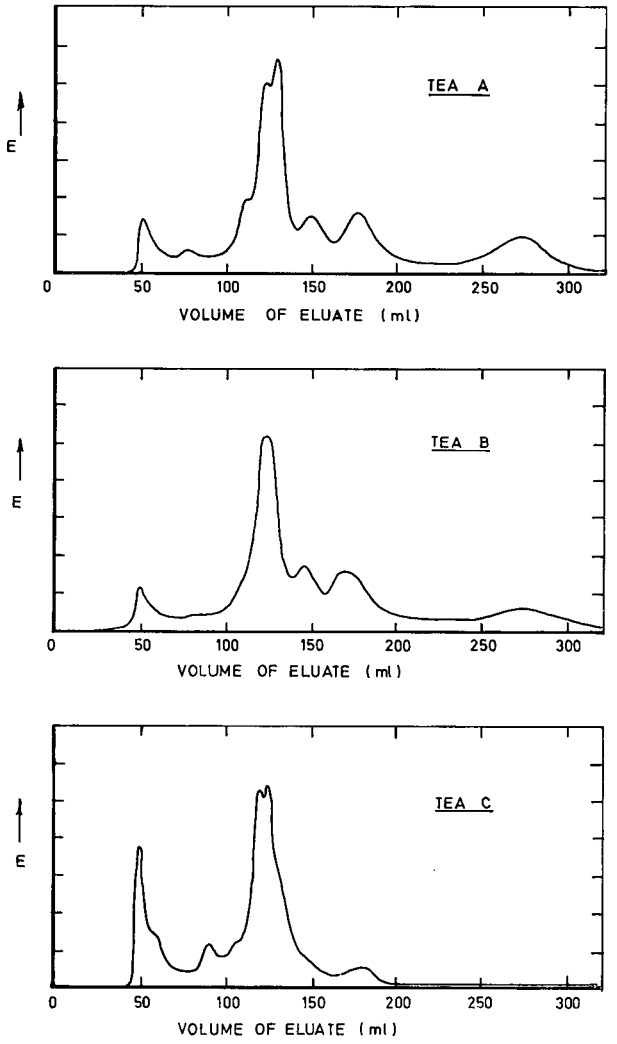

Fig. 1. Elution patterns of three tea extracts.

solution and measured spectrophotometrically. This method is inconvenient and unsuitable for development as an automatic procedure. It has been found that theaflavins can be separated from the other pigments of tea by chromatography on Sephadex LH-20 and this technique is not only suited for automatic operation but is capable of giving additional information on the brown pigments of tea[4] (the so-called "thearubigin" fraction).

Materials and methods

The Sephadex LH-20 gel (Pharmacia Ltd.) was equilibrated in a Whatman (Reeve Angel & Co., Ltd.) chromatography column (31.5 × 2.54 cm) with 60% aqueous acetone. The tea infusion, prepared under standard conditions, was freeze-dried and a sample portion (50 mg) was suspended in 60% aqueous acetone. Polysaccharide material was removed by centrifugation and the supernatant applied to the column which was eluted with the same solvent mixture. The eluent from the column was monitored at 382 nm by passage through a flow-through cell contained in a Vitatron photometer unit (Fisons Scientific Apparatus Ltd.) connected to a logarithmic-scale recorder.

Results and discussion

Elution diagrams for three different teas are shown in Fig. 1. On the column used in this investigation the elution volume[5] for theaflavins is 270 ml and it is clear that tea C which is known to be of very poor quality contains no theaflavins. This is in contrast to teas A and B, which are of good and intermediate quality respectively. At present only the theaflavins peak can be interpreted and it is unlikely that absolute values can be calculated for substances under the peak, since different teas contain varying proportions of theaflavin and theaflavin gallates. The remainder of the elution pattern which is different for each tea, illustrates the complexity of the brown colouring matter of tea. Of particular interest is the observation that these coloured materials are probably eluted from the column in order of decreasing molecular size[4] similar to the behaviour of Crataegus condensed tannins on Sephadex LH-20[6]. The fact that tea C contains a much larger amount of high molecular weight material than tea A confirms BRADFIELD's finding[7] that good quality teas contain less high molecular weight material than those of inferior quality. As further knowledge of the chemical composition of the various coloured fractions and their contribution to quality becomes available, this technique should be a useful aid to evaluating the characteristics of tea liquors.

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CHROM. 3684

Sintered glass as a permanent medium for thin-layer chromatography

Powdered glass has been used as an adsorbent and as a partition support in column chromatography, and by several workers including BRUD[1] as a medium for TLC, by coating a plate in the usual manner by means of a slurry.

Porous glass (Corning Glass Co.), in the form of a powder with a very high degree of microporosity, has been used by KRAMER *et al.*[2] in the same way. MACDONELL AND WILLIAMS[3] successfully separated coloured ink fractions on polished sheets of porous glass, using aqueous solvents and long elution periods. The separate zones were then capable of characterization by light absorption methods through the transparent glass. Activation was produced with hydrofluoric acid and with boiling methanol.

In the present preliminary work, fine powdered soda glass was fractionated by water flotation, retaining a particle size in the range 180 to 220 mesh. A water slurry was spread on glass, allowed to settle, and dried. The layer was sintered by heat, obtaining bonding between the particles and to the supporting clear glass sheet base. The resulting thin-layer plates are resistant to coating removal by accidental abrasion, and can be cleaned by chromic acid or any other normal glass cleaning method, without damage.

Activation of the surfaces of the layer was achieved by treatment with sulphuric acid and heating, and, less successfully, with hydrofluoric acid, phosphoric acid, and methanolic potassium hydroxide, respectively. The detailed procedure giving best activation to date was as follows:

(1) The layer, of 200 mesh soda-glass sintered to soda-glass sheet by heating at 850° for 5 min, resulting in a thickness of 50 to 100 μ, was degreased and cleaned with detergent followed by 0.5% sodium edetate solution, then repeated alcohol and water washing.

(2) The surface was immersed in concentrated sulphuric acid at 100° for 30 min, then washed in distilled water until neutral to blue litmus paper touched to the surface, and finally dried at 150° for 2 h.

Plates prepared in this way are capable of separating the components of the Desaga dye mixture (butter yellow, Sudan red G and indophenol) by eluting with hexane for 2 to 3 min. Some separation was apparent using benzol, but the small size of the plates prepared at this stage did not allow for long elution distances.

Partial success was also achieved in the analysis of a mixture of alkaloids containing morphine, codeine, heroin, quinine and caffeine, on a sintered plate prepared as described, and eluted with benzol. Three distinct zones appeared after spraying with Dragendorff reagent, and a fourth, an overlap, probably quinine, was visible under U.V. light. The plate in this case was made from a microscope slide, and the time of elution was less than 3 min.

An advantage of the proposed layers is their permanence. The surfaces, after the preparation described, appear to retain their characteristics, and therefore produce highly repeatable results, for long periods and many experiments. This may help overcome the variations inherent in normal thin layers due to operational techniques when preparing the plates.

The Stahl dye mixture was also separated on a glass plate which had been

scribed, with a diamond-point, with twenty parallel and closely-spaced lines forming a strip 2.5 mm wide. The amount of material separated was minute (about half a microgram), and examination with a magnifier and coloured filters was necessary to observe the coloured zones. Activation of the glass was carried out in the same way as that of the sintered layers.

Sintered layer plates might also be used as support for a stationary phase in partition separations, with some advantages over analogous paper chromatography.

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CHROM. 3686

Quantitative determination of saturated triglycerides in fats

During the partial catalytic hydrogenation of edible oils, trisaturated glycerides are formed in varying amounts. The extent of formation of these high melting glycerides is not rules by chance, but governed by the hydrogenation conditions. In order to study the parameters which influence their formation there was needed a practically suitable and quantitatively reliable method for the determination of these glycerides. The method described below, based on a TLC–GLC procedure, has been shown to fulfil these requirements.

The classical method is based on a wet oxidation procedure with $KMnO_4$ originally described by HILDITCH AND LEA[1] and later modified by others[2,3] and recently reviewed by CHAKRABARTHY AND GAYEN[4]. The procedure is rather time consuming and the precision and accuracy may, no doubt, be questioned depending on the quantitatively unreliable reactions and manipulations involved. ESHELMAN AND HAMMOND[5] have critically studied the method and conclude that it does not give satisfactory results. Recently another method has been published[6], based upon mercury adduct formation, separation of the non-adduct-forming saturated glycerides and subsequent gravimetric determination.

The present procedure is based upon thin-layer separation of the saturated glycerides on silver nitrate–silica gel coated plates followed by gas chromatographic analysis of the saturated triglyceride fraction after its conversion to methyl esters. The quantification is accomplished with the aid of an internal standard consisting of a suitable saturated triglyceride, which is added to the sample *before* the thin-layer chromatographic procedure. The standard thus accompanies the sample throughout the analysis.

EXPERIMENTAL

Thin-layer chromatography. Glass plates, 20 × 20 cm, coated with Silica Gel G (Merck AG, Darmstadt, Germany) containing 12.5% $AgNO_3$, 0.5 mm in thickness. About 25–50 mg of the sample was dissolved in a chloroform solution containing a suitable (see below) and known amount of the standard (glycerol triheptadecanoate, Hormel Institute, Austin, Minn., U.S.A.). The solution was applied on the starting line of the plate as a streak together with two spots of the reference mixture. The plate was eluted with chloroform until the solvent front traversed about 15cm. After drying, the chromatoplate was visualized with 2′,7′-dichlorofluorescein. The saturated triglyceride zone was scraped off the plate and extracted with *n*-hexane–diethyl ether (1:1). The solvent was driven off with the aid of nitrogen, and the remaining glycerides transesterified according to a modified method originally described by GLASS AND JENNESS[7].

Gas chromatography:

Instrument: Varian Aerograph 1200.

Column: 6 ft. × $^1/_8$ in. stainless steel, packed with 10% DEGS on Anakrom ABS 70–80 mesh.

Temperature: injector, 270°; column, 175°; detector, 210°.

Area measurement: disc integration.

Calculation. From the percentage of the methyl esters the ratio methyl heptadecanoate/Σ(saturated methyl esters) was calculated. Based upon the known amount of glycerol triheptadecanoate added to the sample, the total amount and percentage of saturated triglycerides originally present in sample could then be calculated.

Results and discussion

From a number of determinations of hydrogenated vegetable oil (softening point*, 38–40°) a mean value of 16.7% saturated triglycerides was found with a standard deviation of 1.7%. By allowing the standard to accompany the sample during the whole procedure, the quantitative reproducibility of the thin-layer procedure is not critical in this respect as long as the ratio of the standard and the sum of saturated glycerides remains constant. The accuracy was further investigated in recovery tests by adding known amounts of a secondary standard. The determining factor for the reliability of the whole analysis is thus primarily the GLC procedure. Taking into consideration the generally accepted precision of the gas chromatographic analysis reported by HORNING *et al.*[8], among others, the above mentioned deviation seems reasonable, since the individual GLC percentage error is roughly doubled in the following ratio calculation. In order to achieve the highest possible precision and accuracy the amount of standard must be chosen in such a way that the ratio falls in the 0.25–4 range, since low percentages of either standard or sample increase the relative error of the GLC analysis, which very considerably influences the ratio and subsequently the precision and accuracy of the total analysis.

Owing to the presence of the standard the quantitative removal of the saturated triglyceride zone is not critical. The contamination of unsaturated glycerides should, however, not be overlooked. Such complications are obviously more frequent in the case of hydrogenated fats, owing to the presence of positional and geometrical isomers. *Trans* double bonds for instance are not so firmly bound to $AgNO_3$ compared to the

corresponding *cis* bonds. In those cases where contamination occurred, which could be seen by the presence of unsaturated fatty methyl esters, the percentage of the sum of saturated fatty esters was corrected by substracting twice the percentage of the unsaturated esters from the above mentioned sum with the assumption that the contaminating glycerides were mono-unsaturated. The empirically corrected values were in agreement with those values obtained without any observed contamination. The general validity of the correction has, however, not been investigated.

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* A.O.C.S. Official Method Cc 3-25.

CHROM. 3715

Estimation of trisaturated glycerides in fats by argentation thin-layer chromatography

The classical methods for the estimation of trisaturated glycerides(GS_3) in fats originally developed by HILDITCH[1] and later modified by KARTHA[2] and VON RUDLOFF[3] were essentially based on the oxidation of the unsaturated portion(GU) of the mixed glycerides. In recent years these methods have largely been replaced by modern chromatographic techniques such as column[4], thin-layer[5] and gas–liquid[6] chromatography, by which rapid and accurate determination of triglyceride composition has been achieved.

For the evaluation of mixed glycerides the application of argentation thin-layer chromatography, initially developed by DE VRIES[5], has been well established. This method is based on the separation of mixed glycerides into components according to the degree of unsaturation, for example GS_3, GS_2U, GSU_2 and GU_3, and also according to the isomeric configuration of unsaturated glyceride molecules. Taking advantage of this principle, quantitative methods for the analyses of both natural and synthetic triglycerides have been developed. Thus BARRETT *et al.*[7] have separated mixed glycerides of a number of natural fats on a silver nitrate-impregnated silica gel plate using chloroform–acetic acid–ethanol as solvents. They subsequently estimated the

individual glyceride components by first charring the spots with phosphoric acid and then employing densitometric measurement. Other workers[8–10] have utilised the same principle in separating various natural and synthetic triglycerides on a preparative scale (80–100 mg) on a thick-layer of adsorbent using benzene–diethyl ether, etc. as solvent. The triglyceride components thus separated in band form were detected under ultraviolet light after the application of a dichlorofluorescein spray and subsequently extracted from the adsorbent with dry ether. Each fraction was then analysed either by a titrimetric[8] or colorimetric[9,10] determination of the glycerol liberated from the triglyceride moiety. Among the recently developed micro techniques[11,12] for the quantitative estimation of triglycerides, the method of VAN HANDEL[13] is found to be widely used.

The present work was undertaken to develop a micro argentation thin-layer chromatographic method for estimating in a single spot the trisaturated glycerides in fats of biological importance.

Experimental

The materials under investigation were standard tripalmitin (Hormel Institute, U.S.A.), refined and bleached groundnut oil fortified with known amounts of pure tripalmitin, coconut oil, groundnut oil randomised with 15% pure trimyristin, commercially available butter, butter fat(ghee) and hydrogenated groundnut oil. For the present study, these samples were prepared as 1% solutions in dry benzene, except for coconut oil which was 0.5%.

The solvents and other reagents used were all of analytical grade. Diethyl ether was made peroxide-free by washing repeatedly with saturated solutions of $FeSO_4$, followed by distilled water, and then being dried over a mixture of anhydrous Na_2SO_4 and fused $CaCl_2$, and finally being distilled at a constant boiling temperature.

All-glass apparatus including scratchless and uniform glass plates (10 × 20 × 0.2 cm) were thoroughly cleaned and washed with chromic acid and distilled water and finally dried.

The preparation of a silver nitrate–Silica Gel G layer of 325 μ on plates has been reported elsewhere[14].

Definite volumes in μl (Lamda pipette, Fisher, U.S.A.) of standard tripalmitin solution were taken in glass-stoppered test tubes (16 × 2 cm) and after the removal of the solvent on a water bath, the glycerol content was estimated according to VAN HANDEL *et al.*[13] as follows: μg amounts of tripalmitin left in the test tubes were saponified with 0.5 ml of 0.4% ethanolic (aldehyde-free absolute ethanol) KOH at 60–70° for 30 min, and acidified with 0.5 ml of 0.25 *N* H_2SO_4. After the complete removal of ethyl alcohol on a boiling-water bath, 0.1 ml of 0.05 *M* $NaIO_4$ was added and mixed. Ten minutes later 0.1 ml of 0.5 *M* Na_2AsO_3 was added and allowed to stand for 10 min. Five ml of chromotropic acid, prepared by mixing in the cold an aqueous solution of sodium salt of chromotropic acid (0.448 g sodium salt of chromotropic acid in 40 ml distilled water) with 180 ml of sulphuric acid (120 ml conc. H_2SO_4 mixed with 60 ml distilled water), was then added and allowed to develop colour for 30 min on a boiling-water bath. After the solution had cooled, the optical density was measured at 570 mμ against the "reagent blank" with the help of a Zeiss or Unicam spectrophotometer.

Artificial mixtures containing groundnut oil and graded amounts of standard tripalmitin were prepared. Known volumes of the solution were spotted quantitatively

on a silver nitrate–Silica Gel G plate with a Lamda pipette. The plate was developed in a chloroform–acetic acid (99.5:0.5, v/v) solvent system. When the solvent front reached 14 cm (45 min), the plate was taken out of the chamber, heated at 110° in an oven for 15 min to remove the solvent, cooled and carefully sprayed with ammonia solution (40 ml of liquor ammonia diluted with 100 ml distilled water) until the layer was uniformly wetted. When GS_3 was identified as a white visible spot near the solvent front, the spot area was marked immediately with a needle. The plate was then air dried for 30 min, heated at 110° in an oven for 15 min and finally cooled at room temperature until the ammonia was removed. The contents in the marked area were then scraped off by means of a scalpel or sharp-edged stainless-steel spatula and put directly into a wide-mouthed miniature column (8 × 0.8 cm) fitted with a grease-free stopcock having a small filter bed (1.5–2 cm length) of acid-washed Celite 545 over a cotton plug. The column had been previously flushed with ether and the filter bed was always kept under ether. Thus tripalmitin was extracted quantitatively with 15 ml ether and the ether eluate was collected in test tubes. After careful removal of the solvent on a warm-water bath, the glycerol content was estimated from the residue as described above. In this case the colour was measured against an "experimental blank" obtained by scraping the portion covering an approximately equal blank area as that of GS_3 from the plate being subjected to similar treatment. A "reagent blank" was also simultaneously made for comparison with the "experimental blank" value.

In all subsequent experiments, the GS_3 content of all the samples was determined accordingly. For each plate and every determination an "experimental blank" and a "reagent blank" were performed.

Results and discussions

By drawing a standard curve with increasing amounts of tripalmitin (μg) against the corresponding values of the optical densities (O.D.), a straight line is obtained as shown in Fig. 1. Deviation was observed, however, only at an appreciably high concentration of tripalmitin.

In all the investigated samples the trisaturated glyceride content determined

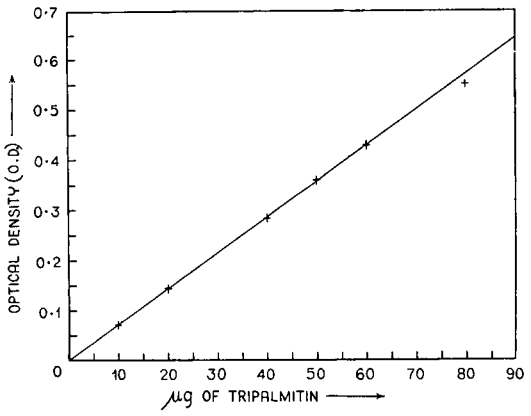

Fig. 1. Standard curve of μg amounts of tripalmitin against the corresponding experimentally determined optical densities (O.D.).

by the present method was derived from the standard curve (Fig. 1) by comparing the corresponding O.D. values measured in each case. The percentage composition was calculated from the known amount of material spotted.

Table I represents known and experimentally determined GS_3 content of groundnut oil mixed with pure tripalmitin. The experimental values are the means of three determinations; two were spotted on a silver nitrate–Silica Gel G plate at equal concentrations (100 μg each of 1% solution) and the other one was spotted at a higher concentration (150 μg of 1% solution). For the experimental value of (a), however, larger amounts (200–250 μg) of a 2% solution were subjected to investigation.

Table II indicates the GS_3 content of other fats. Here also, three determinations for each sample were made according to Table I and the mean values are given. In these cases, however, a sample of groundnut oil containing a known amount of pure

TABLE I

ESTIMATION OF FORTIFIED TRIPALMITIN IN GROUNDNUT OIL MEDIUM BY ARGENTATION THIN-LAYER CHROMATOGRAPHY

Sample	*% Tripalmitin (GS_3)*	
	Known	*Found*
(a)	1	0.98
(b)	3	2.91
(c)	5	4.88
(d)	10	9.92
(e)	30	29.4
(f)	50	49.2
(g)	60	58.2

tripalmitin was also spotted on the plate as "reference sample" and the tripalmitin content was estimated along with each material to study the efficacy of the procedure and its reproducibility. It should be noted that in the case of coconut oil, the amount spotted on the plate for two determinations was 25 μg each and that for one determination was 50 μg.

For the detection of GS_3 on a silver nitrate–Silica Gel G plate, the present method requires aqueous ammonia as a specific spray reagent, although other triglyceride components can also be visualised as faint spots. By using dichlorofluorescein as a spray reagent in the present case, high values with inconsistent results were obtained due to contamination of trace amounts of dye that remained even after extraction of GS_3 through an activated silica gel column.

Further, the use of a small filter bed column of celite was sufficient for the quantitative recovery of GS_3 with ether. A single-packed bed permits about five such successive extractions including the blank from each plate, provided the flow rate (which should be moderately adjusted) is not sufficiently disturbed by the formation of large air bubbles. It may, however, be mentioned that during the removal of ammonia from the plate at 110°, some blackening of spots in the lower region of the chromatogram, consisting mainly of unsaturated glyceride molecules, was observed, whereas the trisaturated glyceride spots remained unchanged.

By comparing the optical densities of a number of "experimental blanks" from

TABLE II

ESTIMATION OF GS_3 IN FATS BY ARGENTATION THIN-LAYER CGROMATOGRAPHY

Sample	*% GS_3*
Groundnut oil randomised with 15% trimyristin	3.0 (2.7)*
Coconut oil	85.0
Commercial butter	13.5
Commercial butter fat (ghee)	18.5
Hydrogenated groundnut oil	10.1

* Denotes the calculated value.

a number of plates measured against "reagent blanks", a positive deviation of O.D. values between 0.02–0.03 was always observed. If the deviation exceeds 0.05, erroneous results may be expected.

A preliminary qualitative study of fat samples by argentation thin-layer chromatography is recommended to adjust the amounts to be employed in the present quantitative micro method in order to obtain well defined spots.

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Quantitative Dünnschichtchromatographie von ^{14}C-markierten Nukleotiden

Die Untersuchung enzymkatalysierter Reaktionen des Nukleotidstoffwechsels erfordert zuverlässige Trennungen sowie empfindliche quantitative Bestimmungen der Nukleotide, besonders wenn die eingesetzten Enzymaktivitäten relativ gering sind. Da bei Enzymtesten in der Regel eine grössere Anzahl von Proben unter identischen Bedingungen untersucht wird, werden einfache und zeitsparende Methoden bevorzugt. Daher ist die Ionenaustauschchromatographie auf der Säule für diesen Zweck wenig geeignet. Die Trennung von Nukleotiden auf der mit PEI-Cellulose beschichteten Platte bzw. Folie jedoch, wie sie K. RANDERATH UND E. RANDERATH eingeführt haben[1], erwies sich uns als zuverlässige Methode. Voraussetzung allerdings war, dass störende Salzeffekte, die aus der biologischen Probe herrührten, bei der Chromatographie vermieden wurden und dass wir eine empfindliche und wegen der grossen Probenzahl relativ einfache Methode zur quantitativen Bestimmung der ^{14}C-markierten Nukleotide verwenden konnten.

Material und Methoden

Dünnschichtchromatographie auf Polygramfolien CEL 300 PEI der Fa. Macherey und Nagel, Düren. Alle Reagentien waren p.A. und stammten von der Fa. Merck, Darmstadt, die Nukleotide von der Fa. Boehringer und Söhne, Mannheim, die Desoxyribosenukleotide von Sigma Chemical Company, St. Louis. Die ^{14}C-markierten Nukleotide wurden von NEN—Chemicals GmbH, Dreieichenhain, gekauft. Die Trennung der Nukleotide führten wir nach den Angaben von K. RANDERATH[2] durch. Zur Radioaktivitätsmessung wurden der Dünnschichtscanner der Fa. Berthold, Wildbad, mit dem Messplatz LB 242 K und der Tricarb 3365 der Fa. Packard benutzt. Für die Messung im Flüssigszintillationsspektrometer wurden die Proben wie folgt gewonnen: (a) die Celluloseschicht wurde von der Folie dem Chromatogramm entsprechend in 2–3 mm breiten Zonen abgeschabt und in die Zählgläser überführt, und (b) die Folie wurde dem Chromatogramm entsprechend in 2–5 mm breite Zonen geschnitten und auf den Boden der Zählgläser mit der Schicht nach oben gelegt. Die Messung erfolgte in einem Dioxanszintillatorgemisch mit PPO und Dimethyl-POPOP.

Ergebnisse und Diskussion

Die Bestimmung der Aktivität der CTP-Ribosereduktase[3] im Knochenmark der Ratte[4] setzt die Trennung von CTP und dCTP voraus*. Da aber im Ansatz ein Teil des CTP in CDP umgewandelt wird, ist auch mit der Bildung von dCDP zu rechnen. Die dünnschichtchromatographische Trennung dieser vier Verbindungen stösst jedoch auf Schwierigkeiten und wird daher nach saurer Hydrolyse als CMP und dCMP durchgeführt (Entwickler: 3% H_3BO_3–2 *M* LiCl (2:1, v/v)). Diese Trennung war auch im biologischen Ansatz ohne Schwierigkeiten möglich, nachdem es uns gelungen war, durch Zusatz von EDTA zum neutralisierten Überstand störende Salzeffekte bei der Chromatographie zu beseitigen. Bei Anwendung des Methanolbades

* Verwendete Abkürzungen: CMP = Cytidinmonophosphat; CDP = Cytidindiphosphat; CTP = Cytidintriphosphat; dCMP = Desoxycytidinmonophosphat; dCDP = Desoxycytidindiphosphat; dCTP = Desoxycytidintriphosphat; EDTA = Äthylendiamintetraacetat.

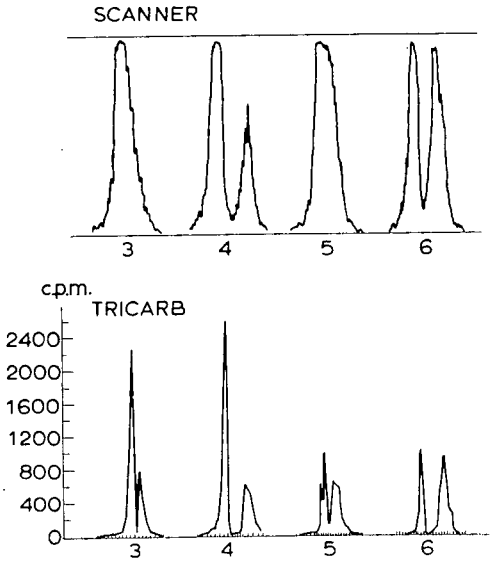

Fig. 1. Kritische Trennung von CMP und dCMP. Abstand der im U.V.-Licht sichtbaren Flecken 2 mm. Untere Reihe: (3) und (4) geschnittene Folie, (5) und (6) abgeschabte Cellulose, Messung im Tricarb. Obere Reihe: die gleichen Proben mit dem Scanner gemessen.

nach K. RANDERATH UND E. RANDERATH[1] war es zu unregelmässigen Verlusten an Radioaktivität gekommen.

Auf der Suche nach einer empfindlichen und relativ einfachen quantitativen Bestimmungsmethode der ^{14}C-markierten Nukleotide standen wir vor folgenden Fragen:

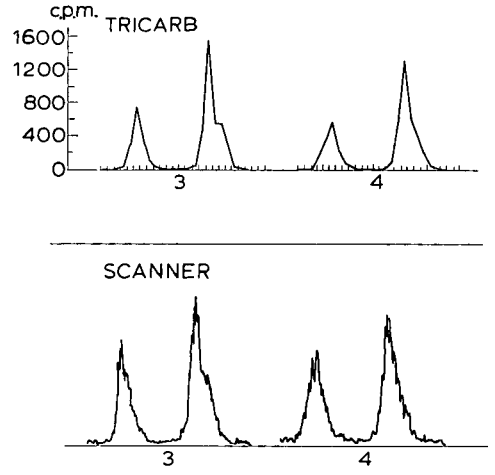

Fig. 2. Gute Trennung von CMP und dCMP. Abstand der im U.V.-Licht sichtbaren Flecken mehr als 2 mm. Obere Reihe: (3) geschnittene Folie, (4) abgeschabte Cellulose, Messung im Tricarb. Untere Reihe: die gleichen Proben mit dem Scanner gemessen.

(1) Ist die Methode des Scannens der Dünnschichtplatte mit dem 2 π-Zählrohr für unsere Zwecke empfindlich und genau genug?

(2) Wie weit beeinflussen Selbstabsorption, geometrische Faktoren und Quencheffekte die Zuverlässigkeit der Messung im Flüssigszintillationsspektrometer, wenn die Proben als abgeschabte Cellulose bzw. als ausgeschnittene Folie in das Zählglas gebracht werden?

Die Impulsraten lagen bei beiden Methoden (des Abschabens der Cellulose und des Ausschneidens der Folie) praktisch gleich hoch. Der einfacheren Handhabung wegen bevorzugten wir die Methode des Ausschneidens der Folie und haben hierbei auch keine Abweichungen durch geometrische Faktoren gefunden, die ihre Ursache im Verschieben der Folie am Boden des Zählglases beim Probentransport im Tricarb hätten haben können. Die Zählausbeute dieser Methode lag bei 85%, wenn wir die Impulsraten der gleichen ^{14}C-Aktivität in Lösung als 100% setzten. Ein Versuch durch Zugabe von Wasser bzw. Trichloressigsäurelösung verschiedener Konzentrationen die 2 π-Geometrie unserer Messanordnung in eine 4 π-Geometrie umzuwandeln, brachte keine wesentliche Steigerung der Zählausbeute. Die Zählausbeute der im Tricarb gemessenen Proben war zehn mal so hoch wie die des Scannens.

In Fällen einer schwierigen Trennung mit einem Abstand der im U.V.-Licht lokalisierten Nukleotide von nur 2 mm brachten die Methoden des Abschabens und des Ausschneidens noch eine Auflösung der Peaks (Fig. 1). Bei guten Trennungen

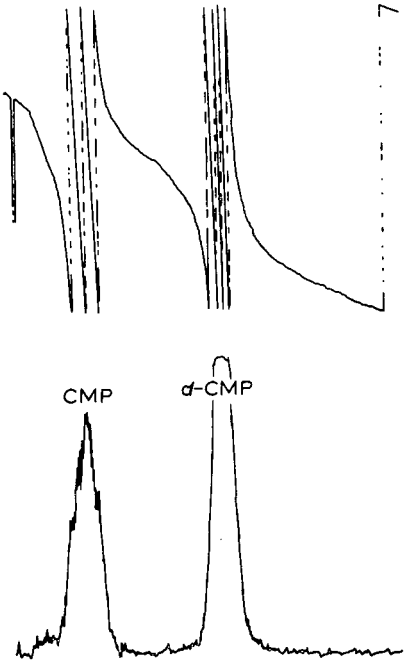

Fig. 3. Scanner Diagramm (DS-Scanner, Fa. Berthold, Wildbad). Vorschubgeschwindigkeit 120 mm/Std.; integrierender Bereich 1000 Impulse, engste Blende. Gleichzeitige Registrierung der Peak-Lokalisation (unten) und der Integration (oben). Trennung von CMP und dCMP.

jedoch mit einem Abstand der Nukleotide von 2 mm und mehr gab auch die Auswertung im Scanner zuverlässige und reproduzierbare Ergebnisse (Fig. 2 und 3).

Damit bietet sich die Methode des Scannens der Dünnschichtplatte bzw. Folie dem Enzymlabor, das eine grosse Probenzahl auszuwerten hat, als Methode der Wahl an, da sie weniger aufwendig ist als die Methode des Abschabens und des Ausschneidens mit der anschliessenden Auswertung im Flüssigszintillationsspektrometer. Doch wird man bei geringen Impulsraten sowie schlechten Trennungen auf diese Methoden zurückgreifen müssen.

Herrn Prof. K. D. VOIGT (II. Med. Klinik UK. Hamburg-Eppendorf) danke ich, dass er mir die Messung auf dem DS-Scanner ermöglichte. Frl. G. SCHMIDT danke ich für wertvolle technische Mitarbeit, der Deutschen Forschungsgemeinschaft für die Förderung der Untersuchung durch Gewährung einer Sachbeihilfe.

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CHROM. 3678

Loss of C-terminal amino acids by hydrazidation during hydrazinolysis

Since the introduction of the method in 1952[1], hydrazinolysis has been very useful for the determination of the C-terminal groups of proteins and peptides. It has, however, always required correction factors, some of them very large, owing to unexplained low recoveries of many of the freed amino acids (see *e.g.* ref. 2). The origin of some of these losses has been examined here in connection with the study of the C-terminal groups of the calf thymus histones[3].

Experimental and results

The C-terminal amino acids of these proteins are alanine, glycine and lysine, and one estimate of the correction factors for these amino acids in the hydrazinolytic procedure has been made by heating the free amino acids in hydrazine at 100°. Samples were run directly in the amino acid analyser. Other samples were completely hydrolysed in 6 *N* HCl and were found to give the full yield of the parent amino acids taken. Further hydrazinolysed samples were chromatographed on Whatman No. 1 paper in duplicate in two solvents. The spots were detected by the ninhydrin reagent of LEWIS[4], which gives useful colour differences with different amino acids and derivatives, and the duplicate chromatograms were dipped in the reagent of ANDREAE[5], which gives a brilliant blue reaction with hydrazides. The ferric chloride stock solution for this was made up in 0.1 *N* acetic acid to avoid precipitation of hydroxide. The results are collected in Table I.

TABLE I

FORMATION OF AMINO ACID HYDRAZIDES FROM FREE AMINO ACIDS DURING HYDRAZINOLYSIS

In all cases the slower ninhydrin-reacting spots correspond in position to the free amino acids. Distances are measured to the centres of the spots.

Amino acid	*Hydrazinolysis (h at 100°)*	*% hydrazidated*	*Paper chromatograms*			
			Solvent A[a] *(cm moved)*		*Solvent B*[b] *(R_F × 100)*	
			NH[c]	*Hydrazide*[d]	*NH*[c]	*Hydrazide*[d]
Alanine	15	66	15.7; 25	24	54; 61	59
Glycine	22	80	11.2; 21.7	21.7	45; 50	50
Lysine	15	41	5.1; 6.8	7.0	36	36

[a] Butan-1-ol–acetic acid–water (3:1:1, by vol.).
[b] Propan-2-ol–0.1 *N* NH_4OH (4.25:1, by vol.).
[c] Ninhydrin reagent[4].
[d] Detected by the ANDREAE reagent[5].

Discussion

It can be seen that an extra ninhydrin-reactive spot is formed in all three cases on hydrazinolysis, and these spots are also strong reducing agents from which the parent amino acids can be regenerated by acid hydrolysis. These extra substances are indistinguishable chromatographically from the hydrazides of these amino acids

prepared by short hydrazinolysis of their esters. The same products also appeared when peptides of these amino acids (Gly–Ala, Leu–Gly and Gly–Lys) were hydrazinolysed for 10 h at 100°, in addition to the expected free C-terminal amino acids.

It is concluded that the major losses of C-terminal amino acids of proteins determined by the hydrazinolytic procedure are due to the formation of the amino acid hydrazides, $NH_2 \cdot CHR \cdot CONH \cdot NH_2$, although with arginine, cysteine, cystine and glutamic acid (which forms pyrrolidone carboxylic acid hydrazide[6]) other considerations also apply. The reaction is not unexpected, since acyl hydrazides such as acetohydrazide can be readily made by heating a mixture of hydrazine and acetic acid without any need to use the ester. It should be mentioned here that a recent excellent paper[7] using catalytic hydrazinolysis at 80° has shown greatly reduced losses of C-terminal amino acids in this method.

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INTRODUCTION

As an instrument in the italian national health policy, the Istituto Superiore di Sanità is charged with the task of disseminating information pertaining to public health. This includes methods of analysis and the purity control of pharmaceuticals.

The present Symposium has been organized to discuss the application of gas chromatography to the analysis of nitrogen-containing drugs used extensively at the present time. Already gas chromatography has revolutionized analytical work in many fields, and it is likely to do the same in the present field by providing a method allowing simpler, faster, and more sensitive checks on purity.

In all its aspects, chromatography offers constantly improving means for investigations in all fields of research and analysis. Gas chromatography has now evolved into a method for analyzing nitrogen-containing organic compounds by its ability to detect very small amounts of amines in animal tissues and biological fluids. Some years ago BECKETT was the first to study the distribution of amines in the body in this way, thus devizing an accurate method of pharmacokinetic investigation. More recently LIBERTI AND CARTONI have analyzed amphetamine in biological fluids and this is of special interest in connection with the problem of doping. STREET has introduced gas chromatography into forensic medicine by applying it to the detection of poisons. Gas chromatography has been used successfully to analyze nitrogen-containing pharmaceuticals by CALÒ AND BONIFORTI and by CARDINI AND QUERCIA in Italy. It has been used as a delicate research tool by CASINOVI, DELLE MONACHE and others of our Institute in the first studies on the separation of the stereoisomers of skytanthine, and by BISSET in work on strychnine alkaloids with a high melting point. The use of gas chromatography has recently been extended from the detection of nitrogen-containing pharmaceuticals in metabolic problems to the quality control and to the quantitative analysis of these preparations. More novelties will no doubt emerge from the following papers and discussions.

I wish to thank again all those who have accepted our invitation, Professors BECKETT and LIBERTI, Doctors BISSET and STREET, and all who left their distant homes to be here today, notably Dr. FORS, the Secretary General of the Nordic Pharmacopœia. My sincere thanks are due to Dr. LEDERER who, as always, helped me with this Symposium, and to Professor RUSSO and his colleagues in the Segreteria didattica for organizing it.

Prof. G. B. MARINI-BETTÒLO

CHROM. 3619

DETECTION OF DOPING BY THIN-LAYER AND GAS CHROMATOGRAPHY

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(Presented December 16th, 1967)

SUMMARY

An analytical procedure for the detection of amphetamine and related drugs in urine is reported. The method consists of a preliminary screening by thin-layer chromatography, followed by scraping the suspicious spots from the plates for confirmation by gas–liquid chromatography on different columns (Carbowax 20 M and Apiezon L). The excretion of amphetamine in man is detectable from 1–2 h after ingestion until 3–4 days later.

In many countries anti-doping control is carried out to prevent the use of various drugs in sporting events. In Italy the Italian Medical Sport Federation (FMSI) undertakes this control, especially in football games and cycle races. Among the drugs most commonly used to improve performance in sport are the amphetamines and related substances. The methods developed for their determination are based on paper[1], thin-layer[2], and gas chromatography[3–5].

The analytical procedure described here, which is used as a routine method for anti-doping control, consists of a preliminary screening by thin-layer chromatography and a subsequent confirmatory test by gas chromatography of a suspicious spot. In this way it is possible to examine a large number of samples in a short time and to support the indication of positive results by gas chromatography with two different liquid phases.

The analyses are carried out on urine, where the unchanged drugs are found at higher concentrations than in the other biological fluids.

EXPERIMENTAL

Extraction procedure

From 5 to 10 ml of urine are placed in a glass-stoppered centrifuge tube and 1 ml of 5 *N* NaOH is added.

The whole is extracted three times with 5 ml of ether with centrifugation always following shaking. The ether extracts are dried over sodium sulphate, transferred to a small test tube having a finely tapered base and then concentrated to 10–15 μl.

Thin-layer chromatography

This technique is used for a rapid analysis of a large number of samples. Only the suspicious spots are scraped from the plate and examined by gas chromatography. For this purpose one must select highly sensitive spray reagents that do not destroy the substances to be tested. Bromocresol green indicator is sensitive to 1 μg of amine and thereby permits recovery of the unchanged drug. This reagent is prepared by mixing a 0.5 *M* phosphate buffer solution of pH 5.5 with a 0.1 % alcoholic solution of the indicator and water in the ratio of 1:2:1.

The plates (20 × 20 cm) are coated with cellulose powder (Whatman CC41) and developed with *n*-butanol–formic acid–water in the ratio of 20:1:2. After drying at 60°, they are sprayed with the above reagent and the spots with R_F corresponding to that obtained for the reference compound are removed for a gas chromatographic confirmatory analysis. Figs. 1 and 2 show plates containing different amounts of amphetamine and some related drugs extracted from urine.

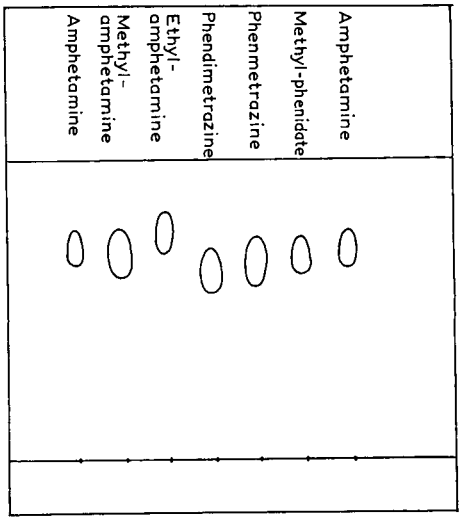

Fig. 1. Thin-layer chromatography of amphetamines and related drugs.

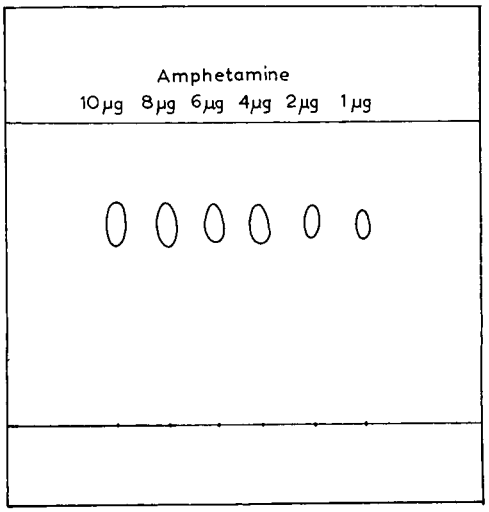

Fig. 2. Thin-layer chromatography of different amounts of amphetamine.

Gas–liquid chromatography

About 1 cm^2 of the positive spots are scraped from the thin layer. The powder is collected in a small test tube, made basic with a few drops of 5 *N* NaOH and extracted as before (three times with 1 ml of ether). The ether extracts, when dried and concentrated, are examined by gas chromatography on two different columns. Glass columns (2 m long, 0.25 cm I.D.) are used on a Carlo Erba (Italy) gas chromatograph model C equipped with a flame ionization detector. The support, Chromosorb 80–100 mesh, washed with 5 % alcoholic KOH solution, is coated with 10 % of the liquid phase. Two different partition liquids are used: a non-polar one (Apiezon L) and a polar one (Carbowax 20 M). Pure nitrogen serves as the carrier gas. The samples plus the standard are injected as solutions in ether (1–10 μl) with a splitter in the ratio of 1:10. Less than 0.1 μg of each amine can be easily detected.

RESULTS

The complete procedure was carried out for some of the amines and the results are reported in Table I. The amine was added to 10 ml of water or urine and, following the procedure described above, the amount recovered was calculated (using N,N-dimethylaniline as internal standard, added to the ethereal extract before the injection).

TABLE I

RECOVERY OF SYMPATHOMIMETIC AMINES AFTER TLC AND DETERMINATION BY GLC

Compound	*μg added*	*μg extracted from water*	*% recovery*	*μg extracted from urine*	*% recovery*
Amphetamine	5	3.7	75	3.0	60
	10	9.0	90	7.4	74
	15	13.2	86	12.8	85
	20	18.2	91	19.5	97
Methamphetamine	10	8.2	82	6.5	65
	20	18.8	94	16.8	84
Phenmetrazine	10	8.1	81	9.0	90
	20	18.0	90	14.0	70
Phendimetrazine	10	8.7	87	6.5	65
	20	16.2	81	16.0	80

TABLE II

URINARY EXCRETION IN MAN AFTER ADMINISTRATION OF 10 mg OF D-AMPHETAMINE

Hours	*ml of urine*	*μg/ml*	*% excretion*
2	140	0.71	1
4	125	1	1.25
8	170	3.79	6.49
12	245	2.18	5.36
16	170	4.61	7.84
20	105	3.81	4.01
24	50	3.56	1.78
28	205	0.31	0.63
32	170	0.44	0.75
36	435	0.04	0.17
40	300	0.04	0.114
44	130	0.05	0.068
48	70	0.03	0.024
52	165	0.02	0.046
56	245	0.02	0.047
60	255	0.03	0.092
64	170	0.02	0.048
68	145	0.03	0.040
72	130	0.002	0.003
76	—	—	—
		Total	29.7

As shown in Table I the method gives a recovery of 60–90 %. These figures can be considered satisfactory because quantitative evaluations cannot be carried out in this anti-doping control since the urinary excretion of the drugs is greatly affected by pH and many other physiological factors.

The method is very specific. When only the "positive" spots obtained from TLC are examined by gas chromatography, the gas chromatograms are simple and the results reliable. After the drug has been well characterized and identified, urinary excretion can be followed directly by gas chromatography, as shown by the data in Table II. These values refer to the amphetamine determination on man following an intake of 10 mg. After 1 h it is already present in the urine and can still be detected 4–5 days later.

With this procedure, which combines the advantages of TLC and GLC, many different amphetamines and related drugs can be detected for anti-doping control. A specific identification excludes the interference of other urinary constituents, making positive results clearly evident.

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CHROM. 3462

FORENSIC PROBLEMS IN THE GAS CHROMATOGRAPHY OF AMINES AND ALKALOIDS

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SUMMARY

Many forensic problems using GLC are similar to those of workers in other fields whose material is of biological origin. This paper is concerned with some of the problems encountered in the analysis of alkaloids by GLC. Because alkaloids are polar compounds, the GLC column used must display minimum adsorption. A method is described whereby adsorption of polar compounds by diatomaceous earth is reduced. It is suggested that this method is superior (especially at column temperatures from 250° to 340°) to methods employing conventional "silanising" procedures.

For identification purposes, on-column derivative formation of some of the alkaloids is described. Reagents used for this purpose are acid anhydrides, ketones and aldehydes. Reactive compounds are characterised by alteration of retention time consequent upon formation of derivative.

Reference is made to the formation of ion-pairs of certain alkaloids and the danger of these ion-pairs not being extracted by certain dilute aqueous acids (*e.g.* HCl) from certain organic solvents (*e.g.* $CHCl_3$).

When columns are prepared as described in this paper, they can be used successfully to analyse *sub-microgram* quantities of a large number of alkaloids ranging in mol.wt. from amphetamine to brucine and including such "difficult" alkaloids as morphine.

I think it is rather unfortunate that the meaning of the word "alkaloid" seems to depend to some extent on the particular scientific discipline to which one belongs. To the pharmacologist for example, an alkaloid is a naturally occurring basic nitrogen-containing compound, whereas to the forensic toxicologist an alkaloid is generally taken to denote any basic nitrogenous compound which can be extracted from aqueous alkaline solution by an organic solvent. This latter definition includes both the "classical" alkaloids of natural origin and the new synthetic compounds. It is this meaning to which I refer when I use the term "alkaloid" in this paper. I should, however, point out that this definition leaves out the quaternary ammonium compounds. It also means that all alkaloids are amines. Not all amines, of course, are alkaloids, because compounds such as adrenaline are water-soluble and are not extracted by immiscible organic solvents.

Many of the problems encountered in forensic work are similar to the problems of workers in other fields who use biological material as their starting point and who are trying to solve their difficulties by employing gas chromatography. One problem which is peculiar to the forensic toxicologist is where the examination of post-mortem samples may be complicated by autolysis when substances may be produced which interfere with the analysis. For example, after gross tissue decomposition, tissues may show the presence of β-phenylethylamine and pyridine. β-Phenylethylamine interferes with the assay of amphetamine by ultraviolet–spectrophotometry but by choosing the correct conditions these compounds can be separated by gas chromatography. Other problems include those associated with a limited amount of sample where one may be searching for submicrogram amounts of alkaloid in gram amounts of material. Sometimes complications arise due to the presence of "other" drugs having been administered in treatment just prior to death. An example of this type is seen in the use of nalorphine as an antidote in morphine poisoning and it is pertinent to mention here that about 4 % of an ingested dose of codeine is excreted as morphine. Gas–liquid chromatography provides an excellent solution to this complication by resolving all three drugs cleanly and quickly.

Whilst on the subject of morphine it will be relevant to refer to what I like to call "amphoteric" alkaloids, of which morphine is an example. Compounds in this group of alkaloids possess both a basic amine and an acidic (usually phenolic) group. Besides morphine, the group includes those alkaloids which have undergone metabolic transformation by microsomal enzymes into phenolic compounds.

These phenolic alkaloids usually undergo further detoxification by forming conjugates with glucuronic acid. But from the analytical point of view the phenolic alkaloids present two problems. First, in order to be able to extract them into an organic solvent the pH of the aqueous phase must be adjusted to a value which lies between the pK_a and the pK_b of the compound, and even then the compound may partition more in favour of the aqueous phase. Second, the compounds are more polar than the parent drug which means that unless the column of the gas chromatograph has been carefully prepared, the compounds will show severe tailing.

However, the *main* problem of the forensic toxicologist is one of *identification* of the poisonous compound or compounds in the biological samples submitted to him for analysis. Only when he is certain what the foreign substances are, can he set about finding out how *much* is present: and this quantitative aspect is generally a simpler problem than the qualitative one. In the rest of this paper I shall describe some of the ways in which we have attempted to solve some of these problems.

By my previous definition, an alkaloid may consist of a primary, secondary or tertiary amine, or combinations of these. Also, drugs which are tertiary amines may be metabolised by demethylation to secondary and primary amines and the possibility exists that each of these in turn may be hydroxylated to phenols.

It is well known that, in most cases, the body's detoxifying mechanisms act on drugs to produce compounds which are more polar than the parent substance. From a gas chromatographic point of view this increase in polarity produces certain difficulties if one wishes to avoid using a multiplicity of columns. For example, using a liquid phase of silicone gum rubber such as SE-30 on a diatomaceous earth support which has been "silanised" with dichlorodimethylsilane, it is quite easy to obtain good peak shapes for imipramine, but the monodesmethyl derivative of this compound shows

some tailing and the didesmethyl compound shows considerable tailing. Our problem thus resolves itself into one of finding a way of reducing the adsorption of the alkaloid by the support material. Two ways of tackling this problem immediately spring to mind. On the one hand, the support material itself may be modified and its polarity reduced, or, on the other hand, the compound being analysed may be modified prior to chromatography by derivative formation. A common example of this latter type is the formation of the alkyl esters of fatty acids. However, in forensic work, it is not always known what the compound *is* which is being chromatographed and for this reason I think it is, in general, preferable to attempt to chromatograph the unmodified compound. Besides this, I believe that ideally one would use a non-adsorbing column and that it is towards this end that we should direct our efforts, even though we may never reach our goal. This leaves us then with our attention focussed on the support material.

It is generally considered that the main forces responsible for adsorption of solutes are the weak VAN DER WAAL'S forces and the stronger forces due to hydrogen bonding. The VAN DER WAAL'S forces are neutralised by all liquid phases and so need not be considered here. In the diatomaceous earth, there will be both Si–OH groups and Si–O–Si groups and, as PALFRAMAN AND WALKER[1] have pointed out these will give rise to two types of hydrogen bond, one where the Si–OH functions as the proton donor in the hydrogen bond and the other where the Si–O–Si group functions as the proton acceptor. From this it would seem that even when the Si–OH groups have been "silanised", there still remains the possibility of adsorption of certain types of compounds, *i.e.* those compounds which can donate a proton to the Si–O–Si group, *e.g.* amines, alcohols and water. According to OTTENSTEIN[2] the hydrogen bond formed from the Si–O–Si group is much stronger than that formed from the Si–OH group. Many workers have made use (probably quite unwittingly) of this fact by coating the silanised diatomaceous earth with a polyhydric alcohol which would hydrogen bond to the Si–O–Si groups and leave a number of C–OH groups exposed. These groups are then probably readily "silanised" by an injection of hexamethyldisilazane into the column. Theoretically then, this type of treatment should reduce adsorption of solutes very markedly and, indeed, with such columns in practice quite symmetrical peaks are obtained for low molecular weight amines. It must be remembered, however, that there still will be a few C–O–Si groups present following the use of the hexamethyldisilazane. Incidentally, in the analysis of amines, it is usual to treat the support with potassium hydroxide because this gives improved results and less destruction of solutes. The use of the alkali seems to be quite empirical and I have not yet heard of a satisfactory explanation of its action. However, when *high* molecular weight alkaloids have to be analysed, the temperature of the column has to be raised often above the temperature at which the polyhydric alcohol is stable—and thus the column is ruined. We have, therefore, in our laboratory concentrated our efforts on the use of more stable liquid phases such as the silicone gum rubbers SE-30 and SE-52. It must also be remembered that there may be adsorbing sites in the system other than those associated with the support material, *e.g.* the metal or glass of the column wall and the injector block.

Previous experiments carried out in my laboratory (MCMARTIN AND STREET[3]) have demonstrated that washing with concentrated hydrochloric acid *does* improve the performance of the diatomaceous earth and that best results are obtained by

boiling the earth-acid suspension. We have also confirmed that treatment of the acid-washed earth with the usual "silanising" agents *does* reduce adsorption. However, when thoroughly dried support material is treated with dichlorodimethylsilane, the results are worse than when *damp* material is used. In other words, the presence of a small amount of water appears to be necessary to produce a satisfactory reduction in the amount of adsorption. Further experiments have shown that diatomaceous earth which has been treated with dichlorodimethylsilane improves on heating. These experiments have been extended to heating of the acid-washed earth coated with silicone polymer SE-30. With temperatures above 300° in the absence of oxygen, improved results are obtained. Extension of this work has resulted in the use of packed stainless steel columns which show very little adsorption even of alkaloids such as morphine[4]. These columns are stable up to at least 320°; they display relatively little "bleeding" even at this temperature; and they are suitable for use with sub-microgram quantities of high molecular weight alkaloids; and they are also suitable, at lower temperatures, for compounds of lower molecular weight, *e.g.* amphetamine.

The gas chromatographs used in our studies were the Model 800, Perkin-Elmer and Model 801 F. & M. instruments, fitted with metal injection ports and employing flame-ionisation detectors. Oxygen-free nitrogen was used as carrier gas in all cases; 6 ft. × 1/8 in. O.D. stainless steel columns were used.

I feel that the preparation of the column packing is so vitally important that I shall describe its preparation in detail. Acid washing of the diatomaceous earth is carried out as follows:

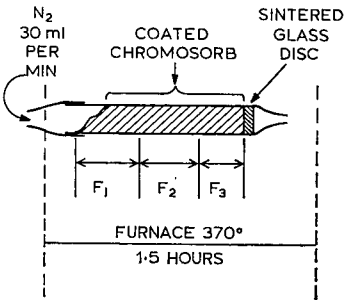
The first stage consists of washing and coating the earth.

About 250 ml of Chromosorb G (100–120 mesh) are washed several times with about 1 l portions of concentrated hydrochloric acid and the powder is then boiled in the acid in a large conical flask for 10 min. The powder is rinsed several times with concentrated hydrochloric acid and then with water until the supernatant liquid is neutral to a pH paper. The suspension of the powder in the water is then boiled for 10 min, rinsed several times with about 10 l of water, the "fines" decanted after each rinsing and excess water removed by vacuum filtration. The powder is placed in flat glass dishes and dried, with frequent stirring, on a boiling water bath.

60 ml of this washed Chromosorb G are put into a 400 ml beaker, 200 ml of toluene are added and the suspension is stirred thoroughly with a glass rod. The powder is allowed to settle and the "fines" are decanted. The washing with toluene is then repeated and as much toluene removed as is possible by decantation. 50 ml of toluene are then added, followed by 100 ml of 10 % water-saturated SE-52 solution and the mixture is thoroughly stirred. Excess toluene is removed by vacuum filtration, and the coated powder is dried in four separate portions with stirring on a hot-plate. The second stage consists of the heat treatment of the coated diatomaceous earth.

The SE-52-coated Chromosorb G is placed in a Pyrex glass tube (see Fig. 1) measuring 2.5 cm in diameter and 40 cm long and fitted at one end with a sintered glass disc. Oxygen-free nitrogen is passed (30 ml per min) through the powder in the glass tube first at room temperature for 5 min and then whilst the tube is heated in a furnace at 370° for 1.5 h. At the end of this period, the tube is removed from the furnace and allowed to cool down to room temperature with the nitrogen flowing. The powder is removed by suction in three separate fractions. These will be referred to as F1, F2 and F3 where fraction F1 is nearest to the nitrogen input. Fraction F1

is then packed into a stainless steel column and the column heated at 370° in a stream of oxygen-free nitrogen (50 ml per min) for 18 h. The column is then emptied, packed with fraction F3, fitted with a short pre-column and heated at 370° in oxygen-free nitrogen (30 ml per min) for 1 h. This packed column is then ready for use.



Heat treatment - silanisation at 370°

Fig. 1. Preparation of column packing. ~~Heat treatment at 370° after silanisation~~.

We have also found that improved results are obtained by first heating the empty steel column in air to a temperature of about 800° (in a furnace) for 18 h.

A possible explanation of the results obtained with our procedure is that the water in the water-saturated SE-52 solution may be required for hydrolysis of the silicone polymer, and the breakdown products so produced, effectively "silanise" the Chromosorb G *at 370°*. It is also possible that a similar process may account for the silicone polymer treatment of the oxidised steel column. Here again, a thermal breakdown product of SE-52 may react with the metal oxide on the inner surface of the metal column to produce a layer which is less polar than the oxide itself. This might then lead to less adsorption and/or less destruction of the compounds being analysed. Such a reaction might also account for the improvement in peak shape when stainless steel columns are heated *in air* prior to packing. The oxide or partial oxide so formed may facilitate subsequent reaction with the thermal decomposition product of the silicone polymer. It is important to note, however, that this thermal breakdown product *must* be formed in the *absence* of free oxygen.

We have also found that steel injector blocks can be treated in a similar manner by injecting a solution of SE-30 or SE-52 into the injector from which the column has been disconnected. The injector should be at about 380° to 400° and, of course nitrogen should be flowing through the block.

Metal systems prepared in this way have proved effective in reducing catalytic destruction of solute. For example, we have found it possible to chromatograph testosterone in amounts down to $5 \cdot 10^{-9}$ g in an all-metal system with a flame-ionisation detector.

Results obtained using one of these columns for a number of alkaloids have been described by STREET[4]. It is also pertinent to note here that the same column can be used for GLC analysis of acidic compounds such as the barbiturates (see MCMARTIN AND STREET[5]) and also of neutral drugs.

Having thus obtained such a column the problem of identification of peaks emerging from the gas chromatograph still remains. In many cases, use can be made of some other parameter such as ultra-violet or infra-red spectroscopy. These may be investigated by extraction of a duplicate sample of original material, or the sub-

stances may be trapped and collected as they emerge from the gas chromatograph. In recent times a lot of attention is being paid to the use of mass spectrometry combined with gas chromatography, and this procedure appears to be proving very successful, although very expensive.

We have been interested in devising a more simple and cheaper procedure for the identification of our peaks. The peak-shift technique, first described by ANDERS AND MANNERING[6] seems to be of value in solving *some* of our identification problems. These workers prepared derivatives on the column by following the injection of the parent compound with an injection of acetic or propionic anhydride.

This procedure enables one to acylate not only both primary and secondary amines, but also any -OH groups present in the alkaloid. We prefer to draw about 3 μl of the anhydride into the syringe, follow this with 2 or 3 μl of the alkaloid solution and to inject this mixture into the chromatograph. Fig. 2 shows the results of this procedure applied to bisnortriptyline.

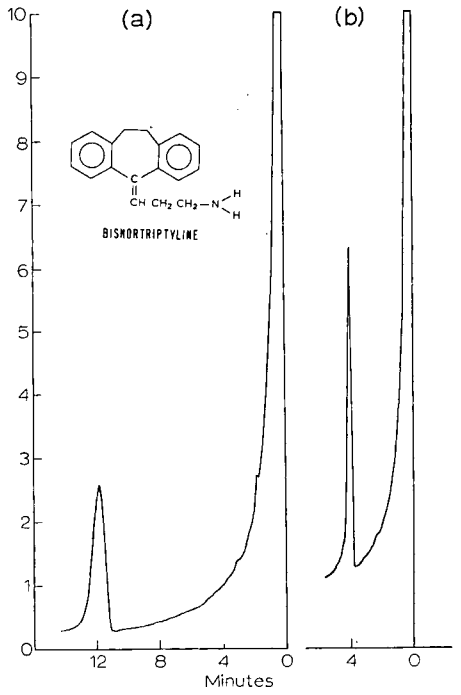


Fig. 2. Chromatograms showing the effect of on-column acetylation of bisnortriptyline, (a) 3 μl Ac_2O + 1 μg (1 μl) of a solution of bisnortriptyline, 250°, attenuation × 10^2; (b) 1 μl EtOH + 1 μg (1 μl) of a solution of bisnortriptyline, 250°, attenuation × 10^2.

In 1965, BECKETT AND ROWLAND[7] made use of a procedure originally suggested by BROCHMANN-HANSSEN AND SVENDSEN[8] in 1962 to identify amphetamine by alteration of its retention time by conversion to its acetone derivative. This they did by first dissolving their residues in acetone prior to injection. However, by using a minor modification, we have found that the residue can be dissolved in ethanol, a portion of this drawn into the syringe, followed by a few μl of acetone, and the acetone–ethanol

mixture injected to produce the same results. The advantage of our modification is that the retention time of the unreacted alkaloid is obtained by direct injection of an ethanolic solution of the residue, and can be followed by the acetone derivative without altering the bulk of the residue solution. This may be important if starting material is limited and if further tests are to be carried out on the residue.

One extension of the peak shift technique which we have found to be particularly useful for high molecular weight alkaloids involves the formation of Schiff's bases on the column. By injecting an ethanolic solution of the alkaloid together with 1 or 2 μl of benzaldehyde (in the same syringe) it is possible to distinguish most primary amines from secondary and tertiary amines. Although, it must be remembered that compounds, such as ephedrine, which display the grouping $>C(OH)-(CH_3 \cdot HN)C<$ will behave as primary amines under these conditions. We have further observed that with a primary amine such as bisnortriptyline, it is not possible to form the acetone derivative on the column at the temperature required to run the alkaloid. This may be because, at this temperature, there is insufficient time of contact between the acetone and the alkaloid to allow reaction to occur to any significant extent. This is supported by the fact that ketones of higher molecular weight *do* react under these conditions.

However, it will be obvious that by a successive use of anhydrides, ketones and aldehydes, much useful information can be obtained by comparing retention times of parent alkaloids and derivatives with reference data.

The condensation product formed from benzaldehyde and amphetamine shows, as expected, that the peak shape of the derivative is more symmetrical than that of the amine, and we are at present working on a method based on the measurement of the area of the peak displayed by the derivative with a view to producing a more sensitive procedure for the quantitative estimation of alkaloidal primary amines in biological material.

BECKETT, TUCKER AND MOFFAT[9] have also made use of derivatives for identification in urine of a number of stimulants. The differences between their work and ours are that their condensation derivatives are formed with ketones whereas we have used aldehydes as well as ketones; the upper temperature limit of their columns appeared to be not greater than 200° whereas we can operate on columns at 320° with only slight increase in background noise. Furthermore our derivatives are formed either in the injector block or on the column. Fig. 3 shows the peak given by the benzaldehyde derivative of bisnortriptyline compared with the peak of bisnortriptyline itself. One μg of bisnortriptyline was used and the column temperature was 270°. The two small peaks are due to an impurity in the benzaldehyde.

BECKETT and his colleagues[9] found that all the primary amines they studied formed condensation products with ketones with the exception of phentermine and chlorphentermine. They suggest that the reason for this may be that the two α-methyl groups in these compounds may hinder the reaction with ketones. We can confirm their findings but it is interesting to note that both phentermine and chlorphentermine *will* condense (on the column) with benzaldehyde—again producing a peak shift.

I would like to mention here the importance in forensic and clinical work of carrying out as extensive an analysis as possible. It may be that a patient is brought into hospital suspected of having taken an overdose of a particular barbiturate. This is mentioned to the analyst who then proceeds to confirm that this barbiturate is in

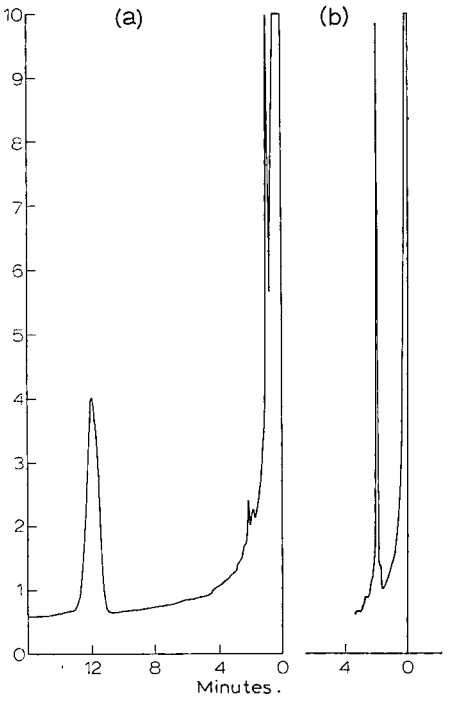


Fig. 3. Chromatograms showing the effect of on-column reaction of benzaldehyde with bisnortriptyline, (a) 1 μl C_6H_5CHO + 1 μg (1 μl) of a solution of bisnortriptyline, 270°, attenuation × 10^2; (b) 1 μg (1 μl) of a solution of bisnortriptyline, 270°, attenuation × 10^2.

fact present. But the blood level of the barbiturate may seem to him to be a little low to be responsible for the patient's condition and, if the analysis had been continued, other drugs may have been found to be present. Imipramine and amitriptyline are drugs which, because of their apparently low concentration, are likely to be dismissed as unimportant even if they *are* found in the blood. We have had post-mortem cases where the blood level of these alkaloids was of the order of 0.5 mg per 100 ml and yet liver levels in these cases were as high as 30 or 40 mg per 100 g.

I think it would not be out of place to refer here to some of the extraction procedures used in forensic toxicological analysis of alkaloids. Generally, one wants to use as rapid a procedure as possible, consistent, of course, with reliability and reproducibility. The usual techniques involve making the fluid or tissue-homogenate alkaline, and then extracting this mixture with an immiscible solvent. Chloroform and ether are the commonest solvents used for this purpose. It is often advantageous to extract the *acidified* tissue with solvent before addition of alkali. This will remove many substances which may interfere with the analysis, *e.g.* acidic and neutral compounds.

Direct extraction procedures of this type are not entirely satisfactory. This is particularly so when the alkaloid is markedly protein-bound. In such cases, direct extraction of protein-rich mixtures may fail completely to reveal the presence of the alkaloid. This failure also applies to procedures designed to remove the protein, by

precipitation or heat-coagulation, followed by extraction of the protein-free filtrate. Here, the bulk of the alkaloid may be left behind attached to the protein.

This difficulty was realised in 1953 by DUBOST AND PASCAL[10] who suggested that the alkaloid should first be released from the protein by brief treatment with hot concentrated hydrochloric acid. I would suggest, therefore, that in the analysis of alkaloids in protein-containing mixtures, this hot acid treatment should be applied. Of course, it must first of all be established by a control experiment that the alkaloid is stable under these conditions. This means that to be of value in an "unknown" analytical search, a list must be compiled of the behaviour under such conditions of all alkaloids likely to be encountered. Even if an alkaloid is *not* stable when treated in this way, the resulting pattern obtained by subjecting an extract of the acid-treated mixture to gas chromatography may be helpful in identification of the drug. As an example of this approach, I would mention that *d*-propoxyphene, which is a tertiary amine, is unstable in hot concentrated hydrochloric acid solution. Subsequent gas chromatography shows instead of the usual peak for the alkaloid, *two* peaks whose retention times might be used to suggest the presence of *d*-propoxyphene in the original mixture.

As regards the *type* of immiscible solvent to be used for extraction, we prefer diethyl ether to chloroform or other halogenated solvents. There are several reasons for this preference. Firstly, it is well-known that certain alkaloids, especially those of the phenothiazine and iminodibenzyl types, can form ion-pairs with a number of inorganic anions at low pH values. These ion-pairs in a chloroform/aqueous acid system, partition very markedly in favour of the chloroform. This means that a drug such as amitriptyline which has, say, been extracted into chloroform from aqueous alkaline solution, *cannot* be transferred from the chloroform by shaking with hydrochloric acid solution. It is very important to note this point. There is at least one recent publication (see FORBES, WEIR, SMITH AND BOGAN[11]) where the authors describe such a procedure for the analysis of amitriptyline in biological material. This is just not possible for amitriptyline itself, and I would suggest that what was actually extracted was probably the mono- or di-desmethyl derivative, *i.e.* one of the metabolites of amitriptyline. We have found that the *primary* amine derivatives do not form ion-pairs under these conditions. A simple change from hydrochloric to sulphuric acid will ensure that the drug *is* extracted from the chloroform. However, we prefer to use *ether* instead of chloroform because the ion-pairs are not extracted by ether.

The *second* reason for preferring ether to chloroform is that ether can be kept more easily in a purified state; it also shows less tendency to emulsion formation; and it is more volatile and therefore more easily removed.

Finally, I want to say a few words about the reporting of results with reference to the amount of drug introduced into the gas chromatograph. Many published articles dealing with gas–liquid chromatography do not give the actual weight of compound introduced into the gas chromatograph. In some cases, it is stated, for example, that 1–3 μl of a 0.5–1.0 % solution of a drug was injected. A tracing of the chromatogram is shown but the reader does not know whether the peak he sees was obtained with 1 μl of a 0.5 % solution or 3 μl of a 1.0 % solution (or any other combination), *i.e.* it is not clear whether 5 μg or 30 μg of drug were responsible for the peak shown. Certain manufacturers too have a habit of showing very tall thin peaks displaying very little tailing and of stating that these peaks were obtained by injection

of "x" μl of solution but without stating the concentration of drug in the solution. The reporting of results in this form is most unsatisfactory; with polar compounds in the sub-microgram region it may be completely misleading. For instance, there is no difficulty at all in obtaining reasonably good peak shapes when 30 μg of morphine are used on some *conventional* columns but 1 μg of morphine usually fails to emerge at all from such columns.

When the weight of compound is not stated it becomes very difficult, and in some cases impossible, to assess the value of other workers' results. This is especially so when, in many cases, the exact details of column preparation are not given. Where amounts *are stated* for alkaloids, these are usually greater than 1 μg and are often of the order of 20 μg. Also, because peak shapes are not shown in many cases, and where they *are* shown tailing may be quite marked, it is unwise to extrapolate to the sub-microgram region. It is my experience that, with *conventionally* "silanised" supports in metal columns, very few high molecular weight alkaloids can be "run" satisfactorily in amounts less than 2 μg. With our columns, a large number of high molecular weight alkaloids can be successfully chromatographed in the sub-microgram region.

ACKNOWLEDGEMENT

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CHROM. 3501

GAS–LIQUID CHROMATOGRAPHY OF TERTIARY *STRYCHNOS* ALKALOIDS

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SUMMARY

The gas–liquid chromatography of tertiary *Strychnos* alkaloids is described. Examination of the alkaloids from *S. nux-vomica* L. has shown that the method can be readily used for the detection and estimation of strychnine in brucine and *vice versa*; a new minor base, icajine, has been detected and isolated.

Alkaloids from *S. icaja* Baill. have also been examined. JAMINET's alkaloid A from this plant has been shown to be a mixture of vomicine and icajine.

Relationships between the relative retention times and chemical structures are discussed.

INTRODUCTION

In 1960 VANDENHEUVEL and co-workers[1] showed that gas–liquid chromatography (GLC) could be applied to the separation of many different types of alkaloids. They used a 6 ft. × 4 mm I.D. column packed with 2–3 % SE-30 (a methylsilicone polymer) on 80–100 mesh Chromosorb W and worked at temperatures mostly between 204° and 222°. Under these conditions strychnine had a retention time of 25.9 min and brucine of 80.0 min.

Later studies have been concerned with the use of GLC in toxicological work. PARKER *et al.*[2] and BROCHMANN-HANSSEN AND FONTAN[3] investigated the application to the separation of alkaloids of other stationary phases, in order of increasing polarity: XE-60 (a cyanosilicone polymer), EGSS-Y (a polyester methylsilicone polymer), and HI-EFF-8B (a cyclohexane dimethanol succinate polyester). It was found that while a polar stationary phase tended to be more selective, the retention times were significantly increased with increasing polarity of the stationary phase. Table I records the retention times of strychnine for these various phases.

Another study[4] with dichlorodimethylsilane-coated Gas-Chrom Q coated with 1 % neopentyl glycol succinate with or without 1 % polyvinylpyrrolidone (PVP) showed that alkaloids containing alcoholic or phenolic groups had their retention times greatly prolonged by the inclusion of PVP and bases with an aromatic ring

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TABLE I

THE RETENTION TIMES OF STRYCHNINE ON COLUMNS WITH STATIONARY PHASES OF INCREASING POLARITY

Borosilicate glass column 3 ft. × 0.07 in., filled with Gas-Chrom P treated with HMDS + 1% stationary phase[3].

Phase	*Temperature (°C)*	*Retention time (min)*
SE-30	225	11.6
XE-60	220	52.9
EGSS-Y	230	75.0
HI-EFF-8B	240	90.2

system were also affected in the same way but to a lesser degree. Thus, without PVP strychnine had a retention time of 59.4 min and with PVP of 97.9 min. Again the application was primarily to toxicological analysis and a wide range of products was examined.

TERTIARY *Strychnos* ALKALOIDS

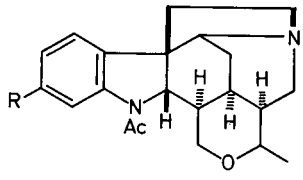
The best known tertiary *Strychnos* alkaloids are strychnine and brucine[5], which occur abundantly in the pharmaceutically important seeds of *S. nux-vomica* L. and *S. ignatii* Berg. Altogether nine tertiary alkaloids, whose formulae are shown in Fig. 1, have been obtained from *S. nux-vomica*, but quantitatively strychnine and brucine predominate by far. Much effort has been directed towards the analysis of these two bases. Many methods have been elaborated, including gravimetric and titrimetric analyses[6], paper chromatography[6,7,23] thin-layer chromatography (TLC)[8], and even nuclear magnetic resonance spectrometry[9]. Analysis of *S. nux-vomica* seeds by paper chromatography or TLC enables strychnine and brucine to be separated, but to do the same for the subsidiary alkaloids may require the use of several different solvent systems.

During studies on *Strychnos* alkaloids, the possibility of applying GLC to the analysis of their mixtures has been investigated. One of the aims was to develop a rapid method for the detection and estimation of small amounts of strychnine in brucine and *vice versa*. Brucine is currently added to denatured alcohol as a tracer and the purity for the brucine must be rigidly controlled. Another aim was to examine the minor alkaloids in the mother liquors from the crystallization of brucine sulphate.

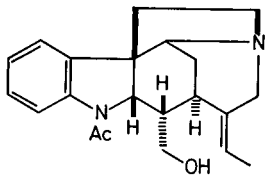
Experimental conditions and retention times

The work discussed in the introduction shows that if brucine and less polar bases like vomicine and novacine are chromatographed on any very polar stationary phase, their retention times will be very long. Such stationary phases would not be suitable for a rapid analysis. The slightly polar SE-52 (a methylphenylsilicone polymer) was therefore tried and proved satisfactory for the purpose in view.

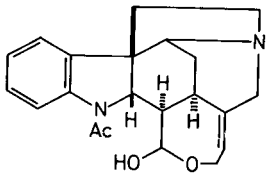
The apparatus used in the experiments was an Aerograph 204–1B, provided with a hydrogen-flame ionization detector (hydrogen flow rate 25 ml/min). The carrier gas was nitrogen at a flow rate of 35 ml/min. The recorder was a Honeywell. A 2 ft. × 1/8 in. Inox (stainless steel) column filled with 100–120 mesh Aeropak 30 coated with



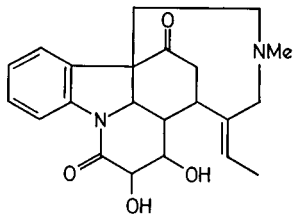
R = H Spermostrychnine
R = OMe Strychnospermine



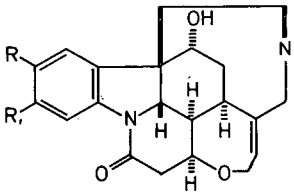
Retuline



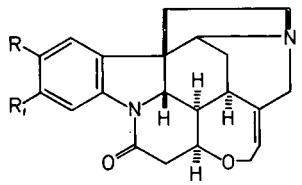
Diaboline



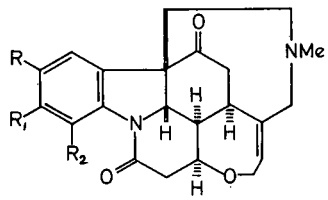
Holstiine ?



R = R_1 = H Pseudostrychnine
R = R_1 = OMe Pseudobrucine



R = R_1 = H Strychnine
R = H, R_1 = OMe α-Colubrine
R = OMe, R_1 = H ß-Colubrine
R = R_1 = OMe Brucine



R = R_1 = R_2 = H Icajine
R = R_1 = H, R_2 = OH Vomicine
R = R_1 = OMe, R_2 = H Novacine

Fig. 1. Tertiary *Strychnos* alkaloids.

5 % SE-52 was used. The alkaloids being examined were injected as 1 % solutions in ethanol, 0.4 μl of solution generally being used. In some of the quantitative work an Aerograph 471 Digital Integrator was available.

Table II shows the retention times relative to strychnine (R_{stry}) and to brucine (R_{bru}) at column temperatures of 230°, 250°, and 280°. The order of elution is the reverse of that on TLC, *i.e.* the more polar alkaloids such as diaboline and retuline have short retention times, while the less polar bases like vomicine and novacine

TABLE II

THE RELATIVE RETENTION TIMES OF TERTIARY *Strychnos* ALKALOIDS

Inox (stainless steel) column 2 ft. × 1/8 in. filled with 100–120 mesh Aeropak 30 coated with 5% SE-52.

Column temperature	*230°*	*250°*		*280°*	
Injector temperature	*260°*	*280°*		*310°*	
	R_{stry}	R_{stry}	R_{bru}	R_{stry}	R_{bru}
Spermostrychnine (324)[a]	0.45	0.44	0.17		
Retuline (338)	0.50	0.49	0.19		
Diaboline (352)	0.76	0.69	0.26	0.69	0.31
Strychnospermine (354)	0.82	0.75	0.29		
Strychnine[b] (334)	1.00	1.00	0.38	1.00	0.45
Holstiine (382)	1.03	0.94	0.36		
α-Colubrine (364)		1.71	0.66	1.59	0.71
β-Colubrine (364)		1.82	0.70	1.66	0.74
Icajine (364)		1.96	0.78	1.88	0.84
Brucine[c] (394)		2.61	1.00	2.24	1.00
Vomicine (380)		3.62	1.41	3.10	1.39
Novacine (424)		5.48	2.18	4.24	2.12

[a] Molecular weight.
[b] Actual retention times: 230°, 11.0, 250°, 5.85, 280°, 2.07 min.
[c] Actual retention times: 250°, 15.2 and 280°, 4.55 min.

have much longer retention times. Since the column is relatively non-selective, the increase in molecular weight will also play a part in increasing the retention times.

A column temperature of 250° gives a satisfactory scale of retention times, as the last base to be eluted, novacine, emerges after about 31 min.

On the other hand, the first six alkaloids, which all have very short retention times, are not all readily separated. Thus, the pairs spermostrychnine[5] and retuline[10–12], diaboline and strychnospermine[5], and strychnine and holstiine[13–15] are incompletely separated from each other. Lowering the column temperature to 210° gives some improvement but β-colubrine is then no longer eluted. The use of a 3-ft. column does not lead to any improvement in the separation either. Not all these alkaloids occur in the same plant at the same time and with the help of TLC it is in fact possible to unravel such a mixture. Further work is clearly necessary to deal with these bases and it may well be that use of a somewhat more polar stationary phase will give the desired separations.

Alkaloids of Strychnos nux-vomica L.

All the *S. nux-vomica* alkaloids—strychnine, α- and β-colubrine, brucine, icajine (N-methyl-*sec.*-pseudostrychnine), vomicine, novacine (N-methyl-*sec.*-pseudobrucine)[5,16]—are well separated with the exception of the two colubrines. The two most important bases, strychnine and brucine, are completely separated, with short retention times and with well-formed peaks which are symmetrical and show no tailing (*cf.* Fig. 2). At a column temperature of 250° strychnine comes out after only 5.85 min and brucine after 15.20 min; at 280° strychnine emerges after 2.07 min

and brucine after 4.55 min. Taking the retention time of strychnine as 1.00, brucine has a relative retention time of 2.61 at 250° and of 2.24 at 280°, which underlines the effectiveness of the separation. The detector responses to strychnine and brucine are in the ratio 100:84 (10 ng strychnine can be readily detected), so that quantitative determinations are readily made.

Clearly, GLC can be of great use in the analysis and purity control of strychnine and brucine.

Isolation of icajine from S. nux-vomica alkaloids

One result of the present work has been the detection in and the isolation from *S. nux-vomica* alkaloids of icajine. The starting material was a partially purified fraction from the mother liquors of brucine sulphate crystallization. The chromatogram of this material is shown in Fig. 2. Peak 2 corresponds to strychnine but together with the little peak 1 in front probably means that some pseudostrychnine or a derivative is present as well (see below). Peaks 3 and 4 belong to the colubrines, peak 5 corresponds in position with icajine, while peaks 6, 7, and 8 represent brucine, vomicine, and novacine, respectively. Small, additional peaks in front of 1, between 2 and 3, and between 7 and 8 may belong to trace amounts of further alkaloids which have not yet been isolated.

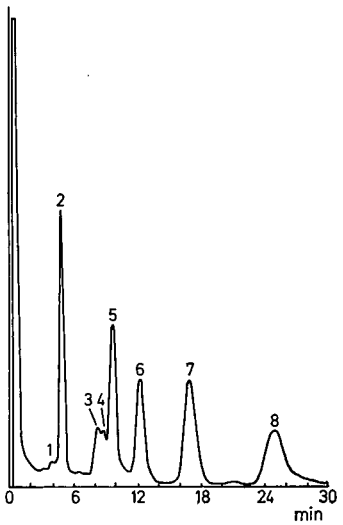


Fig. 2. Chromatogram of a partially purified fraction of the free bases from the mother liquors of brucine sulphate crystallization. 1 = pseudostrychnine subsidiary peak (?); 2 = strychnine; along with peak 1 may indicate that pseudostrychnine is present as well; 3 = α-colubrine; 4 = β-colubrine; 5 = icajine (N-methyl-*sec.*-pseudostrychnine); 6 = brucine; 7 = vomicine; 8 = novacine (N-methyl-*sec.*-pseudobrucine).

With the exception of icajine, all the bases indicated have already been isolated from *S. nux-vomica*. By means of a counter-current distribution between 70% aqueous ethanol and carbon tetrachloride, fractions rich in icajine and vomicine were obtained. Several recrystallizations from methanol eliminated much of the more soluble vomicine, and the icajine was finally obtained pure by preparative TLC.

Comparison with an authentic specimen of N-methyl-*sec.*-pseudostrychnine (m.p. and mixed m.p., optical rotation, TLC and GLC, and infrared spectrum) confirmed the identity[17,18].

The alkaloid has recently been isolated independently by DELLE MONACHE *et al.*[16].

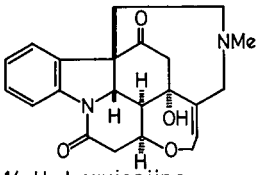
N-Oxides and alkaloids of the pseudo-series

Pseudostrychnine (16-hydroxystrychnine) and pseudobrucine (16-hydroxybrucine) are both known to occur in *S. nux-vomica*[16]. The behaviour on GLC of N-oxystrychnine and pseudostrychnine and its O-ethyl and O-methyl ethers has therefore also been examined.

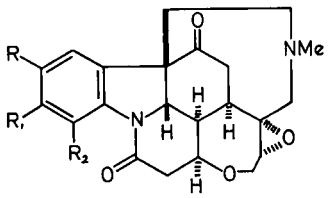
Here again, there have been difficulties, for all of these under the conditions used probably undergo decomposition and in each case the retention time of the major peak is the same as that of strychnine itself. However, in addition to this major peak, N-oxystrychnine shows two small peaks, one on each side of the main one. With pseudostrychnine and its O-ethers, a narrow peak in front of the main one but poorly separated from it is observed.

N-Oxybrucine and pseudobrucine behave similarly.

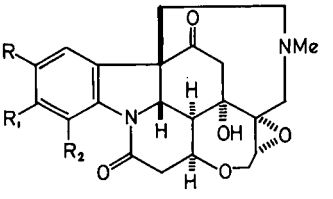
Probably the use of glass injectors and glass columns, rather than stainless steel ones, would reduce the pyrolysis. Perhaps also the use of a more polar column operating at a lower temperature would be advantageous. The point has not been investigated further. However, the complementary application of TLC solves the difficulty, as the N-oxides and derivatives of the pseudo-series are easily separated from the parent bases.



14-Hydroxyicajine
(14-Hydroxy-N-methyl-pseudo-series)



R = R1 = H , R2 = OH
R = OMe , R1 = R2 = H
R = R1 = OMe , R2 = H
(21,22-Epoxy-N-methyl-pseudo-series)



R = R1 = R2 = H
R = R1 = H , R2 = OH
R = R1 = OMe , R2 = H
(14-Hydroxy-21,22-epoxy-N-methyl-pseudo-series)

Fig. 3. Alkaloids from *Strychnos icaja* Baill.

Alkaloids of Strychnos icaja Baill.

This *Strychnos* species, from West and Central Africa, has been, and still is, much used in the preparation of ordeal and arrow poisons[19]. Numerous early investigations of its alkaloids were almost all interpreted in terms of strychnine and brucine. However, in 1951 JAMINET[20] showed by means of paper chromatography that neither of these two bases was present. Later, he[21] reported the isolation of three alkaloids, A, B', and C. A gave two spots on paper chromatography, but on recrystallization appeared not to be altered. Study of it was difficult with the small amount (15 mg) available. The hypothesis that it might be pseudostrychnine, which could conceivably behave as if it were a mixture of carbinolamine and keto-amine forms, was disproved by comparison with authentic material. Professor A. DENOËL, Liège, provided a small sample of JAMINET's product and examination by GLC and TLC suggested that it was a mixture of roughly equal amounts of vomicine and icajine. Separation of the two components by preparative TLC and comparison with authentic vomicine and icajine verified the identities.

Since JAMINET's original work on his alkaloid A, the above two bases have been isolated from the plant and identified[18].

Further alkaloids have been obtained from *S. icaja*[22] and their structures are shown in Fig. 3. So far, representatives of five groups are known and they are in part more complex than those found in *S. nux-vomica*:

(i) pseudo-series (pseudostrychnine, isolated as its O-methyl ether);
(ii) N-methyl-pseudo-series (icajine and vomicine);
(iii) 14-hydroxy-N-methyl-pseudo-series;
(iv) 21,22-epoxy-N-methyl-pseudo-series; and
(v) 14-hydroxy-21,22-epoxy-N-methyl-pseudo-series.

The retention times of these and some related bases relative to that of brucine and at a column temperature of 280° are shown in Table III.

TABLE III

THE RELATIVE RETENTION TIMES (R^{280}_{bru}) of *Strychnos icaja* ALKALOIDS

Inox (stainless steel) column 2 ft. × 1/8 in. filled with 100–120 mesh Aeropak 30 coated with 5% SE-52.

Member	*Series*				
	"Normal"	*N-Me-ψ*	*14-OH-N-Me-ψ*	*21,22-Ep-N-Me-ψ*	*14-OH-21,22-Ep-N-Me-ψ*
Strychnine	0.45 (334)[a]	0.84 (364)	1.00 (380)	—	1.06 (396)
Vomicine	—	1.44 (380)	—	1.57 (396)	1.82 (410)
α-Colubrine	0.71 (364)	—	—	—	—
β-Colubrine	0.74 (364)	—	—	1.49 (410)	—
Brucine	1.00 (394)	2.12 (424)	—	2.28 (440)	2.66 (456)

[a] Molecular weight.

Relative retention times and chemical structure

Examination of Tables II and III reveals some interesting connections between the relative retention times and the chemical structure.

Table II shows the general tendency of higher molecular weight alkaloids to have longer retention times. The first six bases with the exception of strychnine all have an acetyl and/or free aliphatic hydroxyl groups present and are evidently rather polar.

Substitution by methoxyl groups in the aromatic ring as in the series spermostrychnine–strychnospermine, strychnine–colubrines–brucine, etc., leads to notable increases in the relative retention times. On the other hand, on going from the "normal" series to the N-methyl-pseudo-series there is a rather greater increase in relative retention time: R^{280}_{bru} 0.45 for strychnine (mol.wt. 334) increases to 0.71 and 0.74 for the colubrines (mol.wt. 364) but to 0.84 for icajine (mol.wt. also 364).

Vomicine (mol.wt. 380) with its hydroxyl group at C-4 in the aromatic ring forms an exception. It has R^{280}_{bru} 1.44, while the isomeric 14-hydroxyicajine has the much smaller value 1.00. 21,22-α-Epoxyvomicine (mol.wt. 396) has R^{280}_{bru} 1.57, but 21,22-epoxy-N-methyl-*sec.*-pseudo-β-colubrine (mol.wt. 410) has the smaller value 1.49. Here, the differences are not due simply to molecular weight effects. It is known that the phenolic hydroxyl group in vomicine is strongly hydrogen-bonded to the amide carbonyl function[5] and this has the effect of reducing the polarity of the base, as compared with the non-hydroxylated base icajine; this can also be observed in TLC where in methylene dichloride–methanol systems vomicine has a greater R_F value than icajine. Even though at the high column temperature of 280° the intramolecular hydrogen bond is presumably still present, the consequent reduction in polarity is not sufficient to reduce the R^{280}_{bru} value below that of the corresponding higher molecular weight colubrine derivative.

Changes in the alicyclic part of the molecule in the N-methyl-pseudo-series also lead to increasing R^{280}_{bru} values. While epoxidation of the 21,22-double bond brings about a small increase:

Vomicine	1.44	21,22-α-Epoxyvomicine	1.57
Novacine	2.12	21,22-α-Epoxynovacine	2.28,

the introduction of a 14-hydroxyl group, whether the 21,22-double bond is epoxidized or not, causes a somewhat greater increase:

Icajine	0.84	14-Hydroxyicajine	1.00
21,22-α-Epoxyvomicine	1.57	14-Hydroxy-21,22-α-epoxyvomicine	1.82
21,22-α-Epoxynovacine	2.28	14-Hydroxy-21,22-α-epoxynovacine	2.66.

In contrast, epoxidation of the 21,22-double bond, when the 14-hydroxyl group is already present, has only a slight effect:

14-Hydroxyicajine	1.00	14-Hydroxy-21,22-α-epoxyicajine	1.06.

Not all the members of the various series are known, so that it is not easy to make meaningful comparisons. However, the indications available so far suggest that as further data are acquired, it may be possible to obtain hints about the chemical structure of alkaloids belonging to these series from their relative retention times.

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CHROM. 3460

SEPARATION AND DETERMINATION OF IMIPRAMINE AND ITS METABOLITES FROM BIOLOGICAL SAMPLES BY GAS–LIQUID CHROMATOGRAPHY

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(Presented December 15th, 1967)

SUMMARY

A sensitive method for the determination of imipramine and seven of its major metabolites in biological samples is described. The extraction procedures used are based on the partition properties which were determined for each metabolite. The extracted drug and its metabolites are in some cases transformed into derivatives. They are finally separated and determined by gas chromatography. The sensitivity reached is 0.01–0.05 μg, except for 2-hydroxy-desmethylimipramine (0.2–0.3 μg). Examples of analyses using tissues, bile, and liver perfusion medium are given.

Several authors[1–10] have described the determination of imipramine and some of its metabolites in biological materials. Thin layer chromatography lends itself to the detection of all known imipramine metabolites, though the quantitative evaluation methods[11,12] are of limited accuracy. Thin layer chromatography followed by elution and U.V.-spectrophotometry proved not sensitive enough for studies of imipramine metabolism. Gas–liquid chromatography (GLC), due to its high sensitivity, proved to be the method of choice for obtaining accurate values in metabolic experiments. Gas chromatography has been used for the detection of imipramine and desipramine as early as 1961 by GILLETTE *et al.*[13]. More recently gas chromatography has been used for the determination of the antidepressant dibenzepine and six metabolites by LEHNER *et al.*[14] and for phenothiazines by MCMARTIN AND STREET[15].

In addition to GLC procedures this paper also deals with the determination of the partition coefficients of imipramine and its metabolites between organic solvents and aqueous buffers. Based on these values suitable extraction procedures have been developed for tissues, bile, and perfusion medium. Simple methods for the synthesis of derivatives to be used for the final gas chromatography are also described.

MATERIALS AND METHODS

Substances and abbreviations

Imipramine(IP), desmethylimipramine (DMI), desdimethylimipramine (DDMI), 2-hydroxy-imipramine (2-OH-IP), 2-hydroxy-desmethylimipramine (2-OH-DMI),

imipramine-N-oxide (IPNO), iminodibenzyl (IDB), 2-hydroxyiminodibenzyl (2-OH-IDB) were kindly given by Geigy Ltd., Basle, and dibenzepine (DBZ) by Dr. A. Wander Ltd., Berne. All reagents used were analytical grade purity.

Partition coefficients between aqueous and organic phases

Isotonic phosphate buffer[16], pH 7.4, and *n*-hexane or chloroform, respectively, were used. Previous to partition of imipramine or its metabolites, the phases were saturated with respect to each other by shaking for 5 min. The substance was then dissolved in the aqueous phase (5 ml) and shaken with the organic phase (5 ml) in a centrifuge tube (20 ml) for 30 min at 20°. The initial concentration in the aqueous phase and the final concentrations after equilibration were determined with a Unicam U.V.-spectrophotometer SP 800.

The variation of the partition coefficients as a function of pH was determined by using 1,2-dichloroethane, diethyl ether (peroxide-free) and *n*-heptane as organic phases, phosphate buffers (pH 5.0–7.0), borax buffers[17] (pH 7.0–8.8) and glycine buffers[18] (pH 9.0–12.4) as aqueous phases.

Extraction procedures

Imipramine and its metabolites are extracted from tissue homogenates by solvent extraction. IPNO is separated from the mixture since it undergoes pyrolysis to IDB and other products in the gas chromatography column. The isolated IPNO is reduced to IP and determined as such. The remaining metabolites are further separated into non-phenolic and phenolic bases. Dibenzepine (DBZ) is added as an internal standard.

Rat tissues containing imipramine and its metabolites were homogenized in isotonic phosphate buffer, pH 7.4, 1:3, v/v at 0–5° in a Potter-Elvehjem glass homogenizer. Test extractions were carried out with imipramine, imipramine metabolites, and dibenzepine added to the homogenate. For the initial step of extraction the homogenate was brought to pH 10.0 by adding 1 *N* NaOH and concentrated ammonia.

5 ml portions of homogenates of liver, lung, kidney, and fat tissues (pH 10.0) were shaken three times with equal volumes of diethyl ether (5 ml) for 15 min, then centrifuged and the organic phases reextracted with carbonate–bicarbonate buffer[19], pH 10.0. Imipramine-N-oxide was extracted from the aqueous phases by shaking four times with equal volumes of 1,2-dichloroethane. The dichloroethane extracts were dried with anhydrous Na_2SO_4 and evaporated with a stream of nitrogen (99.9 %).

After separation of IPNO the phenolic bases (2-OH-IP, 2-OH-DMI, 2-OH-IDB) and the non-phenolic bases (IP, DMI, DDMI, IDB, DBZ) were separated by the following procedure. The combined ether extracts were shaken three times for 15 min with 3 ml portions of 1 *N* HCl. The acid extract was made alkaline to pH 10.0 with NaOH and ammonia and shaken three times with 3 ml portions of *n*-heptane–isoamyl alcohol (99:1, v/v). The combined organic phases containing the non-phenolic bases were dried with Na_2SO_4 and evaporated with a nitrogen stream.

The phenolic bases contained in the aqueous phase (pH 10) were extracted with three 3 ml portions of ether and reextracted with three 3 ml portions of 1 *N* KOH. The aqueous phase was titrated to pH 10 by adding conc. HCl and then ammonia. DBZ was also added at this stage to serve as an internal standard for the determination of the phenolic bases. The latter were finally extracted from the alkaline phase

with three 3 ml portions of ether. The combined ether phases were dried and evaporated as indicated above.

Brain homogenates, which never contained phenolic metabolites, were shaken for 15 minutes at pH 10–11 four times with equal volumes of *n*-heptane–isoamyl alcohol (99:1,v/v). The combined organic phase was reextracted four times with 4 ml portions of 0.1 *N* HCl. The acid extracts were then adjusted to pH 10 and extracted with four 5 ml portions of 1,2-dichloroethane. The combined organic phases containing the non-phenolic bases were dried and evaporated.

The low blanks with bile and perfusion medium* allowed another simplified extraction method: 2 ml perfusion medium or 0.5 ml bile were titrated to pH 10.0 with NaOH and ammonia and extracted with three 3 ml portions of ether for 15 min. The organic phases were washed with carbonate–bicarbonate buffer pH 10.0 and the aqueous and organic phases processed as described for homogenates, separation into phenolic and non-phenolic groups, however, was not necessary.

The number of extractions (n) required theoretically for an optimum extraction yield of the major imipramine metabolites has been calculated from the distribution coefficients at pH 10 as determined experimentally.

Gas–liquid chromatography

Apparatus. Gas chromatograph Perkin Elmer 801, equipped with a flame ionisation detector (H_2 35 ml/min, air 340 ml/min) and recorder (Honeywell 1 mV, chart speed 16.5 mm/min). The peak areas were evaluated with an electronic integrator (Perkin Elmer D 24). The pyrex glass column (length 2 m, diameter 2.5 mm) was packed with SE-30 (a methyl polysiloxane) on Anakrom ABS. Nitrogen (30 ml/min) was used as a carrier gas. The temperatures of the injector, column and detector were kept at 320, 240 and 260°, respectively. The substances were dissolved in CS_2–$CHCl_3$ (7:3, v/v) and volumes of 0.5–1 μl were injected with a Hamilton syringe.

Sample preparation. The isolated metabolites, with the exception of IPNO, were acetylated in a glass stoppered tube at room temperature by the following procedure: 0.5 ml acetic anhydride and 0.05 ml of dry pyridine were added to the extracts. After 3 h the reaction mixture was evaporated with a stream of nitrogen.

IPNO was reduced to IP with titanium(III) chloride in the following manner: 1 ml of a solution prepared by mixing 5 ml $TiCl_3$ (Merck), 5 ml H_2O and 1 ml conc. HCl was added to 1 ml of the aqueous IPNO solution and heated to 50° for 10 min. After cooling DBZ was added and the pH of the solution was adjusted to 10 by adding 5 *N* NaOH and concentrated ammonia. IP and DBZ were extracted by shaking with two 4 ml portions of *n*-heptane–isoamyl alcohol (99:1, v/v) for 15 min. The organic phase was dried with Na_2SO_4 and evaporated with a stream of nitrogen. This extract was used directly for gas chromatography since the tertiary amines IP and DBZ cannot be acetylated.

Quantitative evaluation. DBZ was used as internal standard. The peaks were evaluated depending on their shape: (1) by the peak height method; (2) by measuring the peak area by the approximation method of CONDAL-BOSCH[20] (height times width in 15 and 85 % of height); (3) by exact integration of the peak area with the electronic

* Medium for perfusion of isolated rat liver: 40 vol. washed bovine erythrocyte suspension, 60 vol. Ringer phosphate–bicarbonate solution containing 1.5 g/100 ml crystallized bovine albumin, 0.4 g/100 ml glucose and 7.5 mg/100 ml aureomycin.

integrator. The absolute amounts were calculated by means of the following formula:

$$m_i = \frac{A_i \cdot Ff^i_{DBZ} \cdot m_{DBZ}}{A_{DBZ}} \ (\mu g)$$

where

m_i = Amount of substance in μg
A_i = Measured value for peak i
Ff^i_{DBZ} = Correction factor for substance i based on DBZ

$$= \frac{m_i \cdot \text{measured value peak}_{DBZ}}{m_{DBZ} \cdot \text{measured value peak}_i}$$

m_{DBZ} = Amount of internal standard DBZ in μg
A_{DBZ} = Measured value for peak_{DBZ}

Retention time t_R, correction factors, and linearity of the flame ionisation detector. The constancy of the retention times of IP, its metabolites, and DBZ was tested under the optimal conditions outlined above. The amounts injected were in the range of 0.05–0.5 μg. The correction factors were based on the internal standard (DBZ) and determined by injecting standard mixtures of IP, IDB, DBZ and the acetylated imipramine metabolites in various concentrations. Each solution was chromatographed three or four times. The correction factors were plotted against the amount of substance. Linearity of the flame ionisation detector is expressed by a straight line parallel to the abscissa.

RESULTS AND DISCUSSION

Partition coefficients: extraction of imipramine and its metabolites from biological material

Table I gives the partition coefficients of imipramine and its metabolites between an aqueous phase, pH 7.4, and *n*-hexane or chloroform, respectively. The substances are listed in the order of increasing polarity.

TABLE I

PERCENTAGE OF IMIPRAMINE AND ITS METABOLITES IN THE ORGANIC PHASE AFTER PARTITION WITH ISOTONIC PHOSPHATE BUFFER pH 7.4

Substance	*n-Hexane*	*Chloroform*
IP	99.4 ± 1.4*	99.9 ± 0.8*
IDB	99.2 ± 1.2	99.6 ± 1.1
DDMI	70.5 ± 2.3	99.1 ± 1.8
DMI	65.1 ± 2.1	98.5 ± 1.6
2-OH-IDB	44.8 ± 2.0	98.0 ± 1.2
2-OH-IP	25.2 ± 2.0	97.4 ± 1.5
IPNO	10.0 ± 2.2	97.2 ± 1.5
2-OH-DMI	6.3 ± 2.0	55.2 ± 2.1

* Standard deviations from 8 experiments.

The partition coefficients indicate the distribution of the imipramine metabolites over a wide range of polarity. The order of the metabolites is identical in both systems used, 2-OH-DMI being the most polar one. The glucuronides of the phenolic metabolites have not been included in our series, however preliminary partition experiments indicate that their polarity is still considerably higher than that of 2-OH-DMI. Fig. 1 shows that an optimal yield of 2-OH-DMI can be achieved by extraction with diethyl ether at pH 10.0 $\pm$ 0.2. Under the same conditions the partition of IPNO, independent of pH, is 71.9 $\pm$ 1.8 % in 1,2-dichloroethane and 7.8 $\pm$ 2.0 % in ether, thus permitting the separation of IPNO.

The minimum number, n, of extractions required for practically quantitative ($>$ 98 %) isolation of the substances (ratio of phase volumes 1:1) is 2 for ether and n-heptane and 2–3 for 1,2-dichloroethane (Table II). The calculated α values given in Table II were confirmed by test extractions from biological materials.

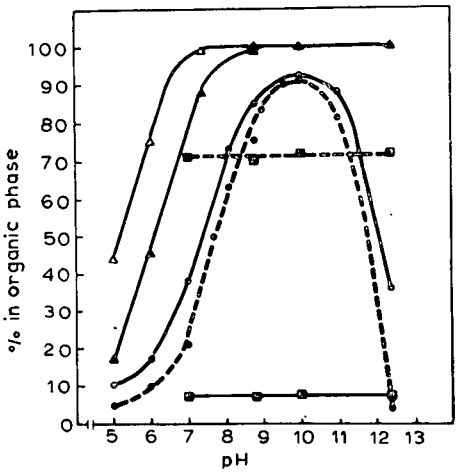

Fig. 1. Partition of IP (△); DMI (▲); 2-OH-DMI (●); and IPNO (■) between aqueous buffer solutions and diethyl ether(———) or 1,2-dichloroethane (– – – –) respectively, as a function of pH value.

Gas–liquid chromatography

The acetylation of DMI, DDMI, 2-OH-IP, 2-OH-DMI and 2-OH-IDB was carried out at room temperature with reaction times from 15 min to 3 h. A maximum yield of 98 % was reached after 2 h. The tertiary amines, IP, IDB and DBZ, are not altered by the procedure.

Anakrom ABS coated with 1.5 % SE-30 shows optimum separation properties (240–260° isothermal). Carbon disulfide proved to be a most suitable solvent for the substances used. The response of the flame ionisation detector to CS_2 is minimal; the peak area of this solvent is 1/100 that of methanol, thus its peak does not interfere with the first peak (IDB) of the analyzed materials. Chloroform has to be added to the solvent since the solubility of the phenolic metabolites in CS_2 is limited. The optimum ratio CS_2–$CHCl_3$ is 7:3,v/v. The solvent properties are hardly altered by the addition of $CHCl_3$ in the ratio mentioned.

TABLE II

MEASURED AND CALCULATED VALUES AS CRITERIA FOR THE EXTRACTION OF IMIPRAMINE AND ITS METABOLITES

Substance	*Diethyl ether*				*1,2-Dichloroethane*				*n-Heptane*			
	% in organic phase	K_c	n^*	α^*	*% in organic phase*	K_c	n^*	α^*	*% in organic phase*	K_c	n^*	α^*
IPNO	7.8 ± 2.0	0.08	2	0.85	71.9 ± 1.8	2.6	3	0.02				
2-OH-DMI	92.9 ± 1.8	13.00	2	0.005	91.1 ± 1.4	10.2	2	0.008	13.8 ± 2.2	0.2	4	0.59
IP	100**				100**				100**			
DMI	98.2 ± 1.5	54.40	2	0.001	98.0 ± 2.0	49.0	2	0.001	98.4 ± 1.4	61.5	2	0.001
DBZ	100**				100**				95.1 ± 2.0	19.4	2	0.002

* $\alpha = \left(\frac{v_1}{v_1 + K_c \cdot v_2}\right)^n$ where v_1 = volume of aqueous phase (5 ml)
v_2 = volume of organic phase (5 ml)
n = number of extractions
K_c = partition coefficient
α = proportion of substance in aqueous phase (pH 10) after n extractions.

** No substance detectable in aqueous phase after one extraction.

The retention times, t_R of imipramine, its metabolites and DBZ are constant in the test range of 0.05–5.0 μg (see Table III). This table also lists the correction factors as based on DBZ. The sensitivity of the flame ionisation detector for all tested substances is linear in the 0.2–3.0 μg range (Fig. 2). Due to the constant capacity of the detector it must be assumed that linearity also exists when samples of less than 0.2 μg are injected.

A standard chromatogram of IP, its metabolites and DBZ extracted from buffer solutions and acetylated is given in Fig. 3. The chromatograms of extracts from

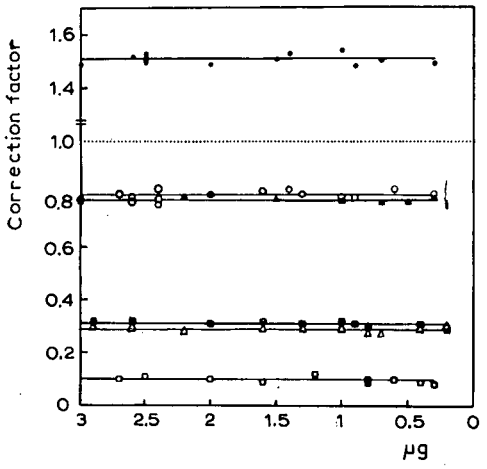

Fig. 2. Correction factors based on DBZ = 1 of IDB (□); IP (△); 2-OH-IDB-Ac (■); 2-OH-IP-Ac (●); DDMI-Ac (▲); and DMI-Ac (O). Linearity of flame ionisation detector.

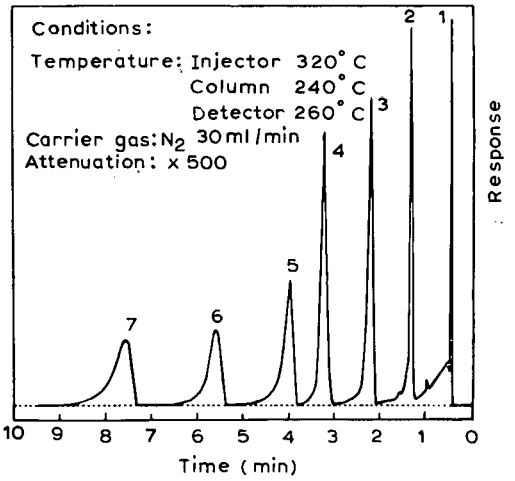

Fig. 3. Standard gas chromatogram. Solvent (1); IDB (2); IP (3); 2-OH-IDB-Ac (4); DBZ (5); 2-OH-IP-Ac (6); and DMI-Ac (7). Peak areas correspond to the amount of substance (1.5–2.5 μg).

TABLE III

GAS CHROMATOGRAPHIC CHARACTERISTICS OF IMIPRAMINE AND ITS METABOLITES

Substance i	*Retention time (min) (column temp. 240°)*	*Correction factor* $Ff^{i}_{DBZ} \pm SD$	*Evaluation method*
IDB	1.30–1.33 (18)*	0.10 ± 0.008 (18)*	peak height
IP	2.18–2.24 (20)	0.29 ± 0.01 (18)	peak height
2-OH-IDB-Ac	3.15–3.21 (18)	0.31 ± 0.01 (23)	peak height
Dibenzepine	3.87–3.93 (20)	1.00	peak height and electronic integration
2-OH-IP-Ac	5.45–5.57 (16)	1.51 ± 0.02 (18)	peak height
DDMI-Ac	6.84–6.90 (16)	0.78 ± 0.02 (14)	electronic integration
DMI-Ac	7.27–7.42 (16)	0.80 ± 0.02 (22)	electronic integration

* Number of experiments in parentheses.

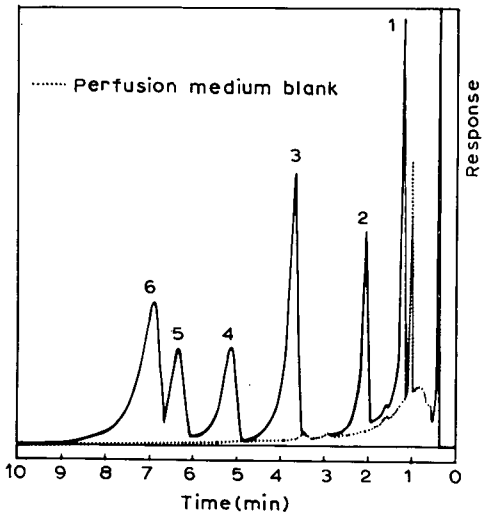

Fig. 4. Gas chromatogram of perfusion medium. IDB (1); IP (2); DBZ (3); 2-OH-IP-Ac (4); DDMI-Ac (5); and DMI-Ac (6); Same conditions as Fig. 3.

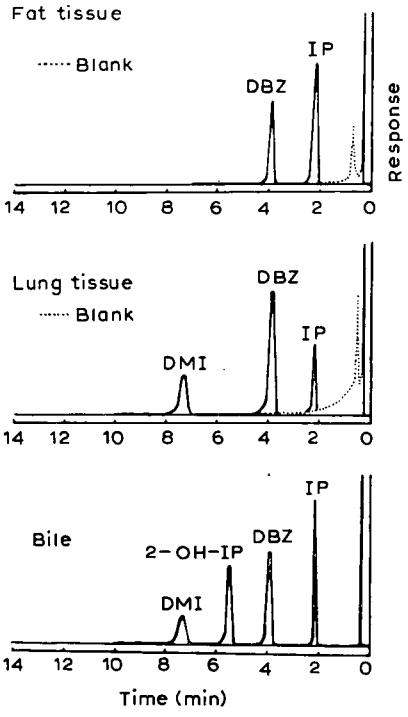

Fig. 5. Gas chromatograms of tissues from rats treated with imipramine, and of bile from a liver perfusion experiment.

biological materials containing no IP or IP-metabolites showed no peaks with retention times identical to those of the mentioned substances. Extracts from liver, kidney and brain showed unidentified peaks in the t_R range of 0–2 min, partially interfering with the IDB peak only. The separation of the peaks of DMI-Ac and DDMI-Ac is incomplete, however graphical evaluation has proved to be satisfactory. Chromatograms of extracts of perfusion medium are shown in Fig. 4; chromatograms from current metabolic experiments in Fig. 5.

The sensitivity of the gas chromatography determination is < 0.01 μg for IDB; 0.01 μg for IP and 2-OH-IDB-Ac; 0.02 μg for DBZ and 2-OH-IP-Ac; 0.05 μg for DDMI-Ac and DMI-Ac. In order to reach these sensitivity values the extracts have to show a high degree of purity. The sensitivity of 2-OH-DMI-Ac is only 0.2–0.3 μg. Since there is considerable tailing of 2-OH-DMI-Ac, an irreversible adsorption of a part of this substance has to be assumed. The retention times of 2-OH-DMI-Ac at column temperatures of 240 and 260° are 11.5 $\pm$ 0.4 and 9.4 $\pm$ 0.2 min, respectively (t_R of DBZ at 260° = 2.15 $\pm$ 0.03 min).

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CHROM. 3589

SYSTEMATIC APPLICATION OF GAS CHROMATOGRAPHY TO THE ANALYSIS OF PHARMACEUTICAL PREPARATIONS

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(Presented December 16th, 1967)

SUMMARY

Gas chromatography has been applied to separate mixtures of psychotropic drugs. A number of monoamine oxidase inhibitors and other stimulating psychotropic drugs (imipramine, phenelzine, methylphenidate, tranylcypromine, pipradol, iproniazid, nialamide and isocarboxazide) was studied. Another group consisting of glutethimide, hydroxyzine, carisoprodol, methaqualone and meprobamate was also examined.

The retention times permit a good separation of these drugs.

Gas chromatographic separation and identification of psychotropic drugs has been studied in recent years with the aim of establishing the metabolism of these substances in the human body, or in order to recognize rapidly drugs used for toxicological purposes[1].

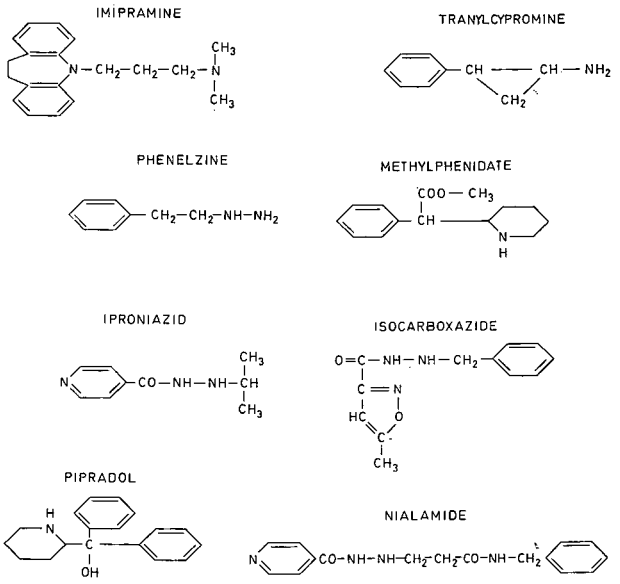

Fig. 1.

The gas chromatographic behaviour of a number of tranquillizers extracted both from phosphate buffer and blood has been studied by this method by NARESH AND KIRK[2], and the relative retention times have been determined.

We have initiated a study of the use of gas chromatography in the analysis of pharmaceutical preparations, both for checking the purity of the substances and for separation and identification of mixtures.

In this preliminary report the separation and identification by gas chromatography of a number of monoamine oxidase inhibitors stimulating psychotropic drugs were studied.

Imipramine, tranylcypromine, phenelzine, methylphenidate, iproniazid, isocarboxazide, pipradol and nialamide were examined. As shown in Fig. 1 all these substances differ in structure but are all characterized by a free amino, hydrazo or hydrazino group.

A good separation of the compounds, with the exception of nialamide, and isocarboxazide, was obtained using the operating conditions given below.

EXPERIMENTAL

Apparatus and materials

Perkin Elmer 801 gas chromatograph, with a differential ionization flame detector.

Glass columns, length 1.80 m and internal diameter 2 mm.

Stationary phase concentration: 2 % GE-SE 30.

Support: Aeropak 30*, 80–100 mesh.

Operating conditions

Glass injector.

Carrier gas flow rate: nitrogen, 50 ml/min.

Operating temperatures: column, programmed 70° to 250° at 10.4°/min; injector 250°; detector 220°.

Method

2.0 μl of a mixture of the above substances in ethereal solution were used. In the mixture the respective concentrations of the substances differed, according to the sensitivity of the material to the conditions used. For quantitative evaluation it was necessary to use a standard chromatogram for all the substances examined.

Under these conditions nialamide and isocarboxazide are decomposed; whereas imipramine, phenelzine, methylphenidate, tranylcypromine, pipradol and iproniazid were separated and detected (Fig. 2).

Another group of psychotropic drugs consisting of glutethimide, hydroxyzine, carisoprodol, methaqualone and meprobamate was examined, although all these substances differ in structure and belong to different chemical groups (Fig. 3).

It was possible to obtain a good separation with the same instrumentation but

* From Varian Aerograph.

with minor variations of the operating conditions (column programmed 100° to 210° at 4.2°/min; injector 200°; detector 230°).

1.0 μl of a 1 % ethereal solution of carisoprodol, glutethimide and methaqualone and 1.0 μl of a 1 % alcoholic solution of hydroxyzine, were injected for this experiment. Meprobamate can be detected, but is partially decomposed.

A good characterization of meprobamate, and separation from other carbamates

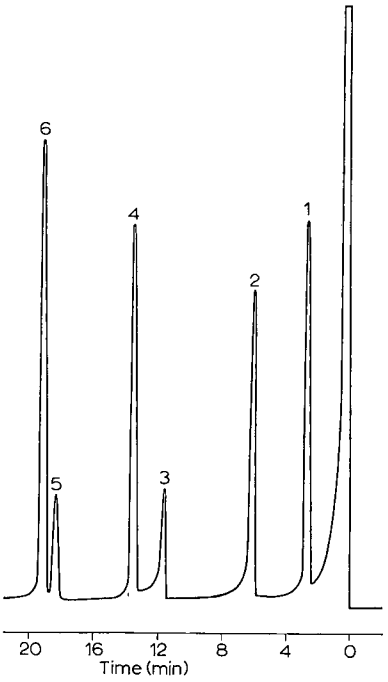

Fig. 2. Separation of a mixture of: (1) phenelzine; (2) tranylcypromine; (3) iproniazid; (4) methylphenidate; (5) pipradol; (6) imipramine.

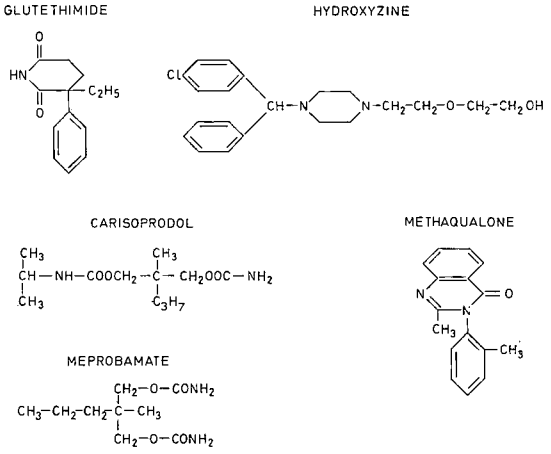

Fig. 3.

such as carisoprodol, mebutamate and tibamate was shown to be possible: the gas chromatographic separation of meprobamate has been studied by DOUGLAS *et al*[3] in plasma and urine, using 1.20 m glass columns containing Diatoport S and 3.8% of methylsilicone VC-W 98; carrier gas flow rate: helium 65 ml/min; detector temperature, 275°; column temperature, 180°.

In our working conditions, the retention times for glutethimide, methaqualone,

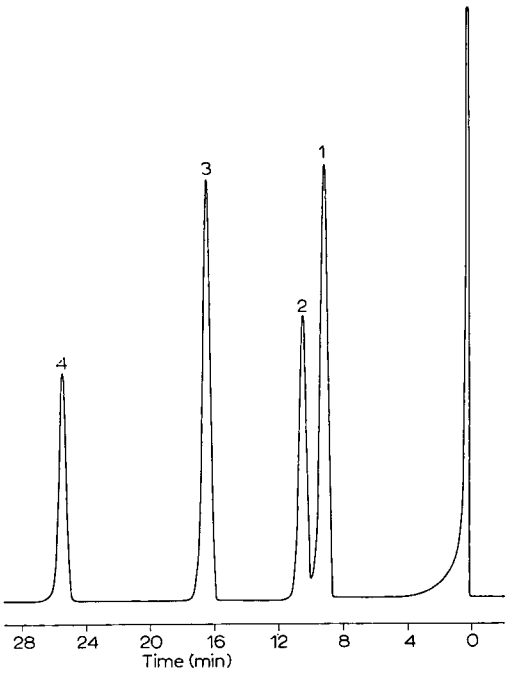

Fig. 4. Separation of a mixture of: (1) glutethimide; (2) methaqualone; (3) carisoprodol; (4) hydroxyzine.

carisoprodol and hydroxyzine permit a good separation of these drugs (Fig. 4). Furthermore, the shape of the peaks also suggests the possibility of quantitative evaluation of these compounds in a mixture.

A limiting factor in the use of GLC is the instability of some drugs to heat, *e.g.* hydrazines, which undergo pyrolysis under the conditions used.

The results obtained indicate that gas chromatographic methods can be used for the evaluation of several psychotropic drugs in mixtures and pharmaceutical preparations.

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CHROM. 3590

GAS CHROMATOGRAPHIC SEPARATION OF SOME ANTITUBERCULAR DRUGS

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(Presented December 16th, 1967)

SUMMARY

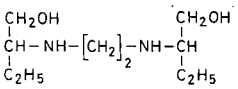
The working conditions for the application of gas chromatography to the separation of some antitubercular drugs in mixtures are described. In particular, ethambutol, iproniazid and isoniazid were studied.

Peaks with good shapes characterized by specific retention times were obtained for each component.

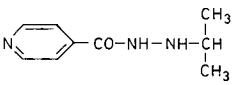
The gas chromatographic separation of several drugs and their determination in pharmaceutical preparations has already been reported from this laboratory[1].

We have now studied the application of this method to the determination of some antitubercular drugs in pharmaceutical preparations and in feeds and foodstuffs. Specifically isoniazid, iproniazid and ethambutol (Fig. 1) have been submitted to gas chromatographic analysis.

ETHAMBUTOL



IPRONIAZID



ISONIAZID

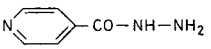


Fig. 1.

Ethambutol has been determined along with other antitubercular drugs in blood, plasma, and urine by colorimetric methods[2] and by microbiological methods[3]. The gas chromatographic behaviour of iproniazid, used earlier as an antitubercular

agent, but now more commonly as an inhibitor of monoamine oxidase, has been studied by CARDINI *et al.*[4]

Good separations were obtained using the following apparatus and operating conditions:

Perkin Elmer 801 gas chromatograph, with a differential ionization flame detector.

Glass columns, length 1.80 m and internal diameter 2 mm.

Stationary phase/concentration: QF1/6 %.

Support: Chromosorb G silanized, 80–100 mesh.

Glass injector.

Carrier gas flow rate: nitrogen, 50 ml/min.

Operating temperatures: column, programmed 120° to 250° at 8.33°/min; injector 250°; detector 250°.

Samples: 1 μl of a 1% benzene solution of TMS ethambutol; 0.5 μl of a 1% alcoholic solution of isoniazid and 1 μl of a 1% ethereal solution of iproniazid were injected.

Peaks with good shapes characterized by specific retention times were obtained for each component. A chromatogram of the separation of the three drugs is shown in Fig. 2.

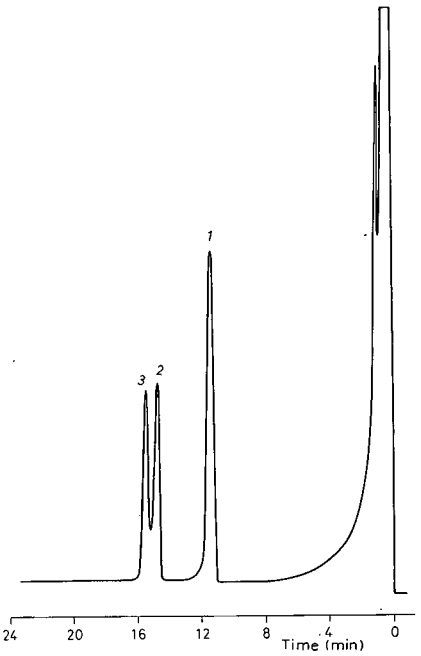


Fig. 2. Separation of (1) ethambutol, (2) isoniazid and (3) iproniazid.

These findings indicate that gas chromatography is an extremely sensitive technique suitable for the detection and determination of these drugs in biological fluids, thus making it possible to study their concentration and metabolic fate in the body. The method could also be useful in the analysis of pharmaceutical preparations

(although antitubercular drugs are seldom found in mixtures), and especially of feeds and foodstuffs containing small quantities of antitubercular drugs. This technique is being extended, both with respect to the compounds mentioned above and to other antitubercular agents.

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CHROM. 3459

CONTRIBUTION A LA SÉPARATION DES AMINES PSYCHOSTIMULANTES PAR CHROMATOGRAPHIE EN PHASE GAZEUSE

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(Présenté le 16 décembre 1967)

SUMMARY

Separation of psychostimulating amines by gas chromatography

The authors propose a gas chromatographic separation method for the five psychostimulating amines: amphetamine, methylamphetamine, phenmetrazine, methylphenidate and ephedrine.

A column of 15 % of XF-1112 on Chromosorb W-HMDS was used as stationary phase and the separation was carried out with temperature programming.

Divers auteurs ont étudié la séparation des amines psychostimulantes par chromatographie en phase gazeuse. PARKER *et al.*[2], BECKETT ET ROWLAND[3], GRECO *et al.*[5], VENERANDO ET DE SIO[4] et VAN ZWOL[10] ont proposé l'utilisation comme phase stationnaire de différents carbowax.

D'autres phases stationnaires—parmi lesquelles le SE-30 et l'Apiezon—ont été proposées par ANDERS ET MANNERING[1], LODI ET MAROZZI[6], KOLB ET PATT[7], LEBBE ET LAFARGE[8] et BROCHMANN-HANSSEN ET FONTAN[9] ainsi que par VAN ZWOL[10]. MOERMAN *et al.*[11,14] ont proposé enfin l'utilisation simultanée d'une phase polaire—le Carbowax 6000—et d'une phase non polaire—le SE-30.

Récemment, nous avons dressé un tableau comparatif des résultats obtenus par ces différents auteurs[14]. Ainsi que nous avons pu le vérifier expérimentalement, aucune des phases stationnaires proposées ne permet une séparation valable des cinq amines psychostimulantes suivantes: amphétamine, méthylamphétamine, phenmétrazine, méthylphénidate et éphédrine.

Dans la présente note, nous rapportons les premiers résultats d'une étude portant sur la séparation, par chromatographie en phase gazeuse, de ces cinq amines psychostimulantes à l'aide d'une phase stationnaire nouvelle—le XF-1112 (Silicone fluid (Nitrile) XF-1112)—et d'une programmation de température. Les essais, actuellement en cours et qui feront l'objet d'une publication ultérieure, permettent d'escompter une application aisée de cette technique à la recherche des amines psychostimulantes dans les milieux biologiques—urines notamment.

* Directeur: Professeur C. HEUSGHEM

CONDITIONS OPÉRATOIRES

Appareil: Aerograph HyFi Model 550-B.
Détecteur: Ionisation de flamme.
Colonne: Verre, longueur: 8 ft., diamètre intérieur: 1/8 in.
Phase stationnaire: 15 % XF-1112 sur Chromosorb W-HMDS.
Températures: Chambre injection: 200°.
Colonne programmée de 60–180° á 4°/min.
Débit azote: 25 ml/min.
Débit hydrogène: 20 ml/min.

RÉSULTATS

Fig. 1 montre la bonne séparation, par la technique proposée, des cinq amines étudiées.

Les limites de détection sont de 0.05 $\mu g/\mu l$ pour la phenmétrazine, de 0.10 $\mu g/\mu l$ pour l'amphétamine et la methylamphétamine, de 0.20 $\mu g/\mu l$ pour le méthylphénidate et de 1.00 $\mu g/\mu l$ pour l'éphédrine.

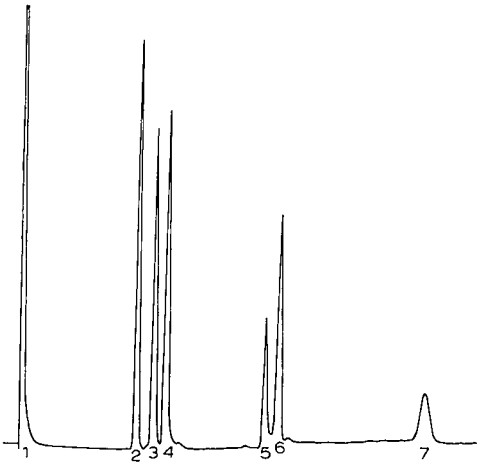

Fig. 1. Séparation des amines psychostimulantes sur XF-1112 en programmation de température. 1 = solvant (éther); 2 = étalon interne (N,N-diméthylaniline); 3 = amphétamine; 4 = méthylamphétamine; 5 = éphédrine; 6 = phenmétrazine; 7 = méthylphénidate.

DISCUSSION DES RÉSULTATS

Ainsi que cela avait déjà été proposé précédemment, la N,N'-diméthylaniline peut être utilisée avec succès comme étalon interne.

Dans les conditions opératoires décrites, aucune interférence n'est à redouter de la part de la caféine, de la strychnine ou de la nicotine. Ce point est important puisque la nicotine est à la base de l'échec des méthodes colorimétriques au méthylorange[12] et des méthodes spectrophotométriques en lumière ultra-violette[13] préconisées jadis pour la détermination des amphétamines dans l'urine.

RÉSUMÉ

Les auteurs proposent une séparation chromatographique en phase gazeuse de cinq amines psychostimulantes: amphétamine, méthylamphétamine, phenmétrazine, méthylphénidate et éphédrine. Pour ce faire, ils utilisent, comme phase stationnaire une colonne de XF-1112 à 15 % sur Chromosorb W-HMDS et opèrent en programmation de température.

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CHROM. 3463

QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF AZAPETINE AND PHENOXYBENZAMINE IN PHARMACEUTICAL PREPARATIONS*

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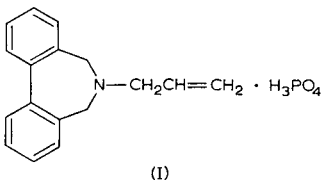
(Presented December 16th, 1967)

SUMMARY

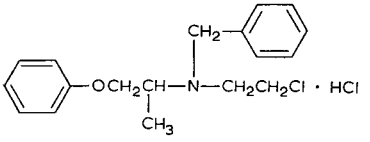
A method is described for the gas chromatographic separation and quantitative determination of azapetine phosphate and phenoxybenzamine hydrochloride in pharmaceutical products in which they may occur separately, together, or in combination with other substances. The stationary phase used was Craig polyester succinate, the internal standard was methyl stearate.

INTRODUCTION

Azapetine (6-allyl-6,7-dihydro-5H-dibenz[*c*,*e*]azepine) and phenoxybenzamine (N-(2-chloroethyl)-N-(1-methyl-2-phenoxyethyl)-benzylamine) are used as vasodilators of the peripheral arteries, the first generally in the form of the phosphate (I) and the second in the form of the hydrochloride (II). Pharmaceutical preparations may contain them singly, together, or in combination with other drugs.



(I)



(II)

A recent publication[1] dealt with the determination of azapetine by spectrophotometry, titration in a non-aqueous medium, and thin-layer chromatography on silica gel. However, the literature contains no specific information on the determination of phenoxybenzamine. It has now been found that gas chromatography permits the rapid determination of these two compounds singly or in mixtures.

Unlike azapetine, it is rather difficult to find the best conditions for the determination of phenoxybenzamine. Notably, the hydrochloride in methanol gives a

* Translated by Express Translation Service, London S.W. 19.

series of peaks due to degradation products, while the amine itself is not sufficiently volatile and gives irreproducible chromatograms. This problem has been overcome by treating the hydrochloride with hot methanolic KOH, whereupon the following reaction takes place:

$$R_1\text{–}N(R_2)\text{–}CH_2CH_2Cl + CH_3O^- \longrightarrow R_1\text{–}N(R_2)\text{–}CH_2CH_2\text{–}O\text{–}CH_3 + Cl^- \quad (1)$$

which has been confirmed by the I.R. spectrum of the methyl ether formed. The latter is sufficiently volatile and stable, and gives rise to a symmetrical and reproducible GLC peak.

EXPERIMENTAL

Apparatus

The Perkin-Elmer 881 chromatograph used was equipped with a double column and a flame ionization detector. The stainless steel columns (length, 46 cm; internal diameter, 0.3 mm) were packed with 80–100 mesh Chromosorb W impregnated with 20 % of Craig polyester succinate (Wilkens) as the stationary phase.

A Leeds-Northrup Speedomax W recorder was used at a full-scale deflection of 2.5 mV, the chart speed being about 5 cm/min. The samples (0.1–0.5 μl) were injected with a 1 μl Hamilton microsyringe.

The column, closed at one end with a suitable metal disk, was attached to a vibrator made in our laboratory. The packing was then introduced with constant shaking before the other end was closed with a similar disk. This part of the column was to be placed in the outlet of the chromatograph. This was repeated with a second column. Both were connected to the glass injection part of the chromatograph, but not yet to the detector, The columns were conditioned by heating at 220° for 12 h in a current of nitrogen (40–50 ml/min) and then for 3–4 h at the same temperature but without the nitrogen, and finally at 185° in a current of nitrogen. The detector was then connected. The internal standard, methyl stearate in petroleum ether, was found to traverse the apparatus in 2–3 min under the conditions used, namely column temperature 185°, temperature of vaporizer 230°, detector temperature 200° and flow rate of carrier nitrogen 30 ml/min.

Quantitative analysis

Principle of the method. Mixtures of known amounts of compounds I, II, and the reference methyl stearate (Q_I, Q_{II}, and Q_{Ref}, in mg) were repeatedly chromatographed, the peak areas (A_I, A_{II}, and A_{Ref}) were measured, and the unit area corresponding to 1 mg of a substance was calculated for all three compounds [A_I/Q_I (= $A_I{}^u$); A_{II}/Q_{II} (= $A_{II}{}^u$); and A_{Ref}/Q_{Ref} (= $A_{Ref}{}^u$)]. This was followed by the calculation of the ratios $A_I{}^u/A_{Ref}{}^u$ (= f_I) and $A_{II}{}^u/A_{Ref}{}^u$ (= f_{II}). The mean values of these f-values for a number of runs (*i.e.* f_I and f_{II}) were then used as calibration factors. The amounts of azapetine and phenoxybenzamine could then be determined by chromatographing them with a known amount of the internal standard, measuring the peak areas, and using the calibration factors.

Preparation of the calibrating mixtures. Each of five calibrating mixtures was

prepared by weighing out accurately about 150 mg of pure azapetine phosphate and about 100 mg of pure phenoxybenzamine hydrochloride. These were placed in a 100 ml ground glass flask, 15 ml of 99.9 % pure methanol were added, and the solution was refluxed for 5–6 min. Then 20 ml of a freshly prepared methanolic solution saturated with KOH in the cold were introduced, and the solution was refluxed for 1 h. The solution was cooled, transferred with the addition of water into a 250 ml separating funnel, and extracted three times with 80, 40 and 20 ml portions of diethyl ether. The extracts were combined, and an accurately weighed-out portion (*ca.* 50 mg) of methyl stearate was added as a reference substance. The solution was shaken with three 15 ml portions of water, and the aqueous layer was discarded on each occasion. The ethereal solution was dried over anhydrous sodium sulphate, filtered through paper into a 250 ml round-bottom flask, reduced in volume to 1–2 ml, and 0.5 ml of toluene added. The sample, which was now ready to be chromatographed, contained methyl stearate, azapetine base, and the methyl ether of phenoxybenzamine formed according to reaction 1.

The resulting chromatogram (see Fig. 1), obtained under the conditions mentioned above, exhibits perfectly symmetric peaks appearing in the following order: (a) solvent, (b) methyl stearate, (c) azapetine, and (d) phenoxybenzamine. Each of the five calibrating samples was chromatographed three times.

Calculation of the calibration factors. The peak area was calculated in mm^2 as height times width at half-height on all 15 charts. The factors f_I and f_{II} were calculated on each chart, and the mean values f_I and f_{II} were found by averaging over

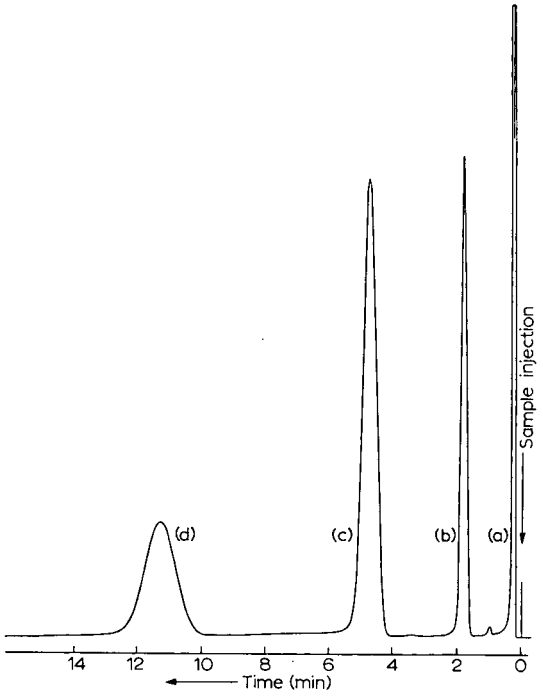


Fig. 1. Chromatogram for a calibrating mixture. a = Solvent; b = methyl stearate; c = azapetine; d = phenoxybenzamine (methyl ether).

TABLE I

EXAMPLE OF THE DETERMINATION OF THE CALIBRATION FACTORS

	Q_I (mg)	Q_{II} (mg)	Q_{Ref} (mg)	A_I (mm^2)	A_{II} (mm^2)	A_{Ref} (mm^2)	f_I	f_{II}
1	152.15	101.15	50.70	307.85	189.00	110.00	0.860	0.805
2				370.70	228.20	142.50	0.867	0.803
3				393.97	243.30	152.30	0.862	0.801
4	150.70	100.30	50.05	878.90	567.00	393.00	0.802	0.794
5				885.50	542.70	364.00	0.808	0.744
6				726.13	477.90	298.10	0.809	0.800
7	150.30	100.90	50.50	922.00	603.00	370.50	0.836	0.810
8				850.11	547.40	342.90	0.833	0.799
9				465.08	293.70	188.50	0.829	0.780
10	150.55	100.50	49.80	285.25	188.65	111.00	0.850	0.842
11				326.25	205.66	125.20	0.862	0.814
12				707.37	465.67	270.20	0.866	0.854
13	150.18	101.66	50.20	370.00	247.95	151.50	0.817	0.809
14				476.46	296.10	194.70	0.818	0.751
15				492.61	322.86	201.30	0.818	0.792
Mean values (f_I and f_{II})							0.836	0.800
Standard deviation							0.023	0.089

TABLE II

Preparation	*Amount of azapetine phosphate per tablet (mg)*		*Amount of phenoxybenzamine hydrochloride per tablet (mg)*	
	Found	*Deviation %*	*Found*	*Deviation %*
Azapetine phosphate tablets (35.4 mg per tablet = 25 mg of pure base per tablet)	34.2	−3.4	—	—
	34.1	−3.6	—	—
	35.5	+0.4	—	—
Phenoxybenzamine hydrochloride[a] capsules, each containing 10 mg	—	—	10.1	+1.0
	—	—	10.2	+2.0
	—	—	10.5	+5.0
Vadigen Gentili[b]	15.4	+2.6	10.3	+3.0
	15.7	+4.6	10.1	+1.0
	15.1	+0.7	10.4	+4.0
	15.6	+4.0	9.8	−2.0
	14.8	−1.3	10.2	+2.0
	15.2	+1.3	9.9	−1.0

[a] Phenoxybenzamine hydrochloride capsules are not produced in Italy.

[b] Each tablet of Vadigen Gentili contained 15 mg of azapetine phosphate, 10 mg of phenoxybenzamine hydrochloride, 50 mg of cyclandelate, and 1 mg of ajmalicine.

all the runs. The more numerous the latter, the better the resulting calibration factors. These must, of course, be determined experimentally whenever the conditions are changed, *e.g.* using a new column or a new batch of methyl stearate. An example of the calculation is shown in Table I.

Analysis of pharmaceuticals. A known amount (P) of a pharmaceutical containing azapetine and/or phenoxybenzamine was treated, if necessary after grinding *e.g.* a tablet of mean weight M, in exactly the same way as the calibrating mixtures, and at least three chromatograms were recorded for each sample. The amount of azapetine phosphate and phenoxybenzamine hydrochloride (mg) present in one tablet was then calculated with the aid of the formulae:

$$\frac{A_{\mathrm{I}} \cdot M \cdot Q_{\mathrm{Ref}}}{f_{\mathrm{I}} \cdot A_{\mathrm{Ref}} \cdot P} \text{ and } \frac{A_{\mathrm{II}} \cdot M \cdot Q_{\mathrm{Ref}}}{f_{\mathrm{II}} \cdot A_{\mathrm{Ref}} \cdot P}$$

where A_{I} is the area of the azapetine peak on a given trace, A_{II} is the area of the phenoxybenzamine peak, A_{Ref} is the area of methyl stearate peak and Q_{Ref} is the amount of methyl stearate introduced (in mg). The resulting values were then averaged over equivalent chromatograms, and some of the data are listed in Table II.

DISCUSSION

The results indicate that the method is suitable for the quantitative determination of azapetine and phenoxybenzamine in drugs, whether they are present in these singly or in mixtures. As in all such work, care is needed in connection with the preparation of the columns, avoiding saturation of these with the compounds to be analysed during the runs, and with the necessity of frequently checking the efficiency of the columns. When care is taken, the results are reproducible with an error of not more than $\pm 5\,\%$.

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CHROM. 3461

GAS CHROMATOGRAPHIC SEPARATION OF 4'-NITROAZOBENZENE-4-CARBOXAMIDES OF PRIMARY AND SECONDARY AMINES

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(Presented December 16th, 1967)

SUMMARY

Primary and secondary amines can be directly separated as 4'-nitroazobenzene-4-carboxamides by gas–liquid chromatography. Using silicone grease as a liquid phase and a constant temperature of 270°, the separation was performed with derivatives of primary and secondary aliphatic, alicyclic, and aromatic amines up to C_{12} and several tobacco alkaloids. A preparation technique is described whereby pure compounds are obtained from complex mixtures ready for mass spectrometric identification. A short review is given with respect to the examination of tobacco and tobacco smoke.

Recent publications have indicated the use of direct gas chromatographic separation techniques for crystallizable derivatives of carbonyl compounds including oximes[1,2] and 2,4-dinitrophenylhydrazones[3–5]. The use of 4'-nitroazobenzene-4-carboxamides (NABSA) for the separation and identification of amines by thin-layer chromatography has been reported earlier[6].

In 1955, the basic reagent 4'-nitroazobenzene-4-carboxylic acid chloride was prepared by HECKER[7], for the first time, and later on used as a reagent for hydroxyl compounds by BUTENANDT *et al.* during investigations on "bombicol"[8,9].

The reaction equations for hydroxyl compounds and primary and secondary amines are shown in Fig. 1.

Derivatives obtained by this method crystallize well, and are strongly coloured substances with exact melting points.

Direct gas chromatography of derivatives proved to be very helpful in the identification of both amines and hydroxyl compounds. Concerning the gas chromatography of hydroxyl derivatives a detailed report will be published, in which retention times of about 100 compounds including alcohols and phenols will be given*.

* This report has been published in the meantime (see ref. 10).

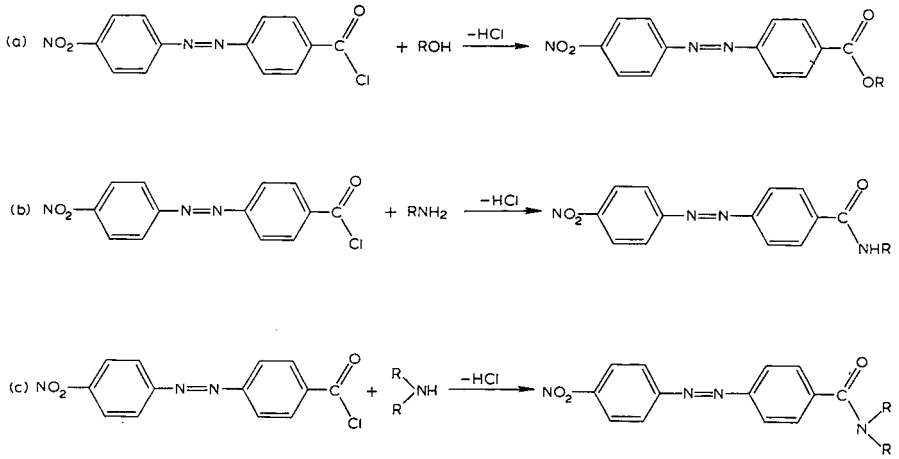

Fig. 1. The reaction equations for: (a) hydroxyl compounds; (b) primary amines; (c) secondary amines.

EXPERIMENTAL

Apparatus

For chromatography, 1-m and 2-m stainless steel tubes (4-mm I.D.) packed with 2.5 % w/w silicone grease (E. Merck AG., Darmstadt, Germany) on 60 to 80 mesh Chromosorb G, acid washed and DMCS treated, were used in fractometers, types F6 and F7 (Perkin Elmer Bodenseewerk, Überlingen, Germany). The fractometers were equipped with flame-ionization detectors and a 2.5-mV recorder (Siemens-Kompensograph L 288 × 288) with a paper feed of 0.5 cm/min.

Conditions

The operations were carried out under the following general conditions:
Constant column temperature: 270°
Injection temperature: 300°
Carrier gas: helium
Flow rate: 30 ml/min, without splitting after the column.

Procedure

The 4′-nitroazobenzene-4-carboxamides of the amines were prepared as described earlier[6] and showed the melting points listed there. Because of their slight solubility the samples were dissolved in dichloromethane and 1 to 5 μg in about 5 μl of the solvent were injected into the apparatus. When a new column is used for the first time or, for example, the injection is interrupted overnight, the column must be pre-loaded with amides in order to get any results. After a number of injections of an amide solution these difficulties were overcome.

Another problem is the small recovery of the injected amides, certainly dependent on the above-mentioned effect; 95 % of the sample seem to decompose while passing through the first part of the column.If a glass column is used a grey zone of 3 or 4 cm length can be seen after one or two injections. Nevertheless, the columns are normally useful for about 4–6 weeks.

A slight peak distortion caused by some bleeding of silicone grease, due to the high temperature used, and deposition of silicone dioxide on the FID electrode, was overcome by using a relatively high hydrogen flow rate to the FID (about 30 ml/min).

Preparation technique

In spite of the great loss of derivatives after injection mentioned above, a preparation technique to obtain traces of pure compounds for mass spectrometric measurements was developed.

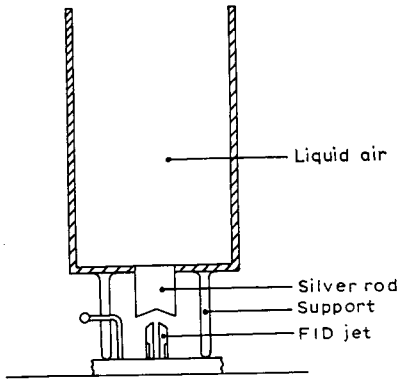

Fig. 2. Plan of the arrangement for obtaining traces of pure compounds.

Fig. 2 gives a key plan of this arrangement. After extinguishing the FID flame the opposite electrode was turned away from the FID jet in the manner shown in Fig. 2; the compounds can then be condensed into the recess of a liquid air cooled silver rod, according to their predetermined retention times.

The condensed derivatives can be recovered by means of fine glass wool moistened with acetone and directly inserted into the sample glass of the mass spectrometer. The smallest sample size injected which gives a distinct mass spectrum is about 5 μg.

RESULTS AND DISCUSSION

Fig. 3 shows the gas chromatographic separation of the NABSA of primary amines. The separation of the *n*- and *iso*-forms is only possible up to C_4, while *n*- and iso-pentylamine cannot be separated.

Fig. 4 gives a corresponding fractogram of the symmetric secondary amines. These secondary amines indicate the increasing influence of the larger molecules with respect to the separation of the *n*- and *iso*-forms.

Fig. 5 shows the fractogram of a mixture of some asymmetric secondary amines.

Fig. 6 shows the slope of the retention times of primary and secondary *n*-alkylamines plotted against the number of C-atoms. It is evident that symmetric secondary amines have much lower retention times than derivatives of primary amines containing the same number of C-atoms. The decreasing effect of methyl and ethyl groups in asymmetric secondary amines is not as strong as that produced by

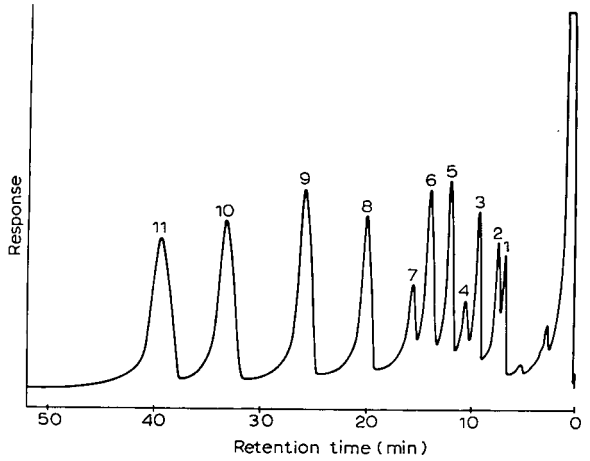

Fig. 3. Gas chromatographic separation of the NABSA of primary amines, using general conditions. 1 = Methyl + ethylamine; 2 = isopropylamine; 3 = *n*-propylamine; 4 = isobutylamine; 5 = *n*-butylamine; 6 = *n*- + isopentylamine; 7 = isopentenylamine; 8 = *n*-hexylamine; 9 = *n*-heptylamine; 10 = *n*-octylamine; 11 = *β*-phenethylamine.

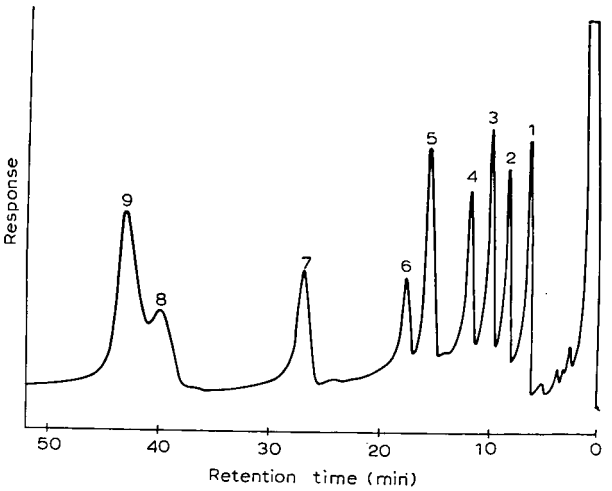

Fig. 4. Gas chromatographic separation of the NABSA of symmetric secondary amines, using general conditions. 1 = Dimethylamine; 2 = diethylamine; 3 = diisopropylamine; 4 = dipropylamine; 5 = dibutylamine; 6 = diisopentylamine; 7 = dipentylamine; 8 = dicyclopentylamine; 9 = dihexylamine.

the symmetric substitution resulting in a medium position of their slopes not given in Fig. 6.

Table I lists the retention times of all the derivatives tested on the 2-m and 1-m columns under the conditions mentioned above. The retention time of the reagent 4′-nitroazobenzene-4-carboxylic acid chloride is 3 min, thus excluding any interference with the derivatives.

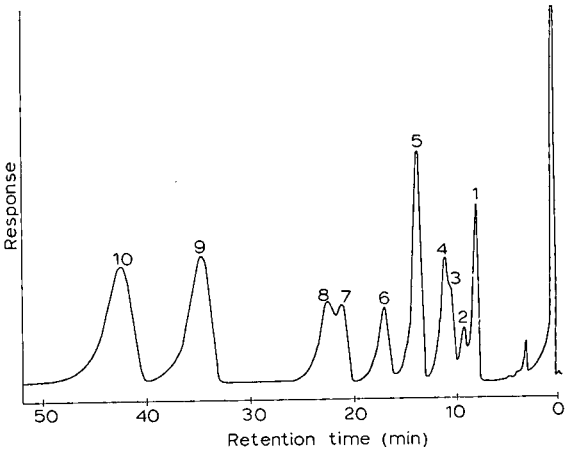

Fig. 5. Gas chromatographic separation of the NABSA of asymmetric secondary amines, using general conditions. 1 = Dimethylamine; 2 = N-methyl-ethylamine; 3 = N-methyl-isopropylamine; 4 = N-methyl-propylamine; 5 = N-methyl-butylamine; 6 = N-methyl-pentylamine; 7 = N-methylaniline; 8 = N-ethylaniline; 9 = N-methyl-benzylamine; 10 = N-methyl-N-β-phenethylamine.

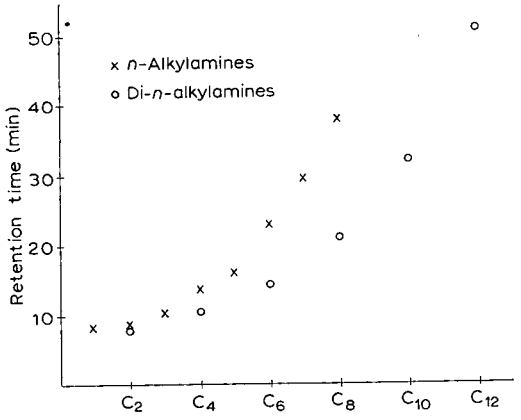

Fig. 6. Retention times on a 2-m column plotted against the number of C-atoms (conditions given in Experimental section).

One can distinguish the derivatives of primary and secondary amines by treatment of the mixture with nitrous gases, after which the derivatives of the primary amines disappear whilst those of the secondary amines still persist.

A mixture of two primary and two secondary amines is shown in the fractogram of Fig. 7; the same mixture is shown in Fig. 8 after treatment with nitrous gases for about 40 min.

Finally, the separation of basic constituents of tobacco and tobacco smoke will be briefly reviewed. Tobacco[11] and tobacco smoke[12] were examined for volatile

TABLE I

RETENTION TIMES OF THE NABSA OF VARIOUS AMINES

2-m column

Parent amine	*Retention time (min)*
Methylamine	8.0
Dimethylamine	8.0
Ethylamine	8.2
N-Methyl-ethylamine	8.6
Diethylamine	10.4
n-Propylamine	10.4
N-Methyl-propylamine	12.8
N-Ethyl-propylamine	12.6
Dipropylamine	14.4
Isopropylamine	8.4
N-Methyl-isopropylamine	11.0
N-Ethyl-isopropylamine	12.0
Diisopropylamine	12.2
n-Butylamine	13.8
N-Methyl-butylamine	14.2
N-Ethyl-butylamine	15.2
Dibutylamine	21.2
sec.-Butylamine	11.2
N-Methyl-*sec.*-butylamine	12.6
N-Ethyl-*sec.*-butylamine	13.8
Di-*sec.*-butylamine	18.4
Isobutylamine	13.0
N-Methyl-isobutylamine	12.6
N-Ethyl-isobutylamine	13.6
Diisobutylamine	17.4
tert.-Butylamine	9.0
N-Methyl-*tert.*-butylamine	11.2
N-Ethyl-*tert.*-butylamine	12.6
n-Pentylamine	16.8
N-Ethyl-pentylamine	17.6
Dipentylamine	32.2
α-Ethylpropyl-amine	14.8
2-Methyl-butylamine	16.2
1,2-Dimethyl-propylamine	13.4
Isopentylamine	16.2
N-Ethyl-isopentylamine	16.4
Diisopentylamine	25.8
n-Hexylamine	23.4
Dihexylamine	51.6
n-Heptylamine	29.6
n-Octylamine	38.0
Allylamine	10.8
N-Ethyl-allylamine	11.6
Diallylamine	14.0
2-Butenylamine	14.6
3-Methyl-2-butenylamine	18.8
Diisopentenylamine	17.8
2-Methoxy-ethylamine	14.0
2-Amino-ethanethiol	9.8
Cyclopropylamine	13.4
Cyclopropane-methylamine	16.4
Cyclopentylamine	21.6

(*continued on p. 211*)

TABLE I (*continued*)

2-m column

Parent amine	*Retention time (min)*
Cyclohexylamine	27.4
3-Cyclohexen-1-yl-amine	28.1
Dicyclopentylamine	46.6
N-Methylaniline	20.8
N-Ethylaniline	22.6
N-Butylaniline	30.8
Diphenylamine	64.0
o-Toluidine	21.2
m-Toluidine	45.0
p-Toluidine	47.4
Benzylamine	39.0
Pyrrolidine	18.2
Piperidine	20.0
2-Methylpyrrolidine	17.4
2-Pipecoline	22.0
3-Pipecoline	20.6
4-Pipecoline	22.6
2-Aminopyridine	28.5
3-Methylamino-pyridine	25.4
2-(Methylaminomethyl)-pyridine	33.4
4-(Methylaminomethyl)-pyridine	41.2
Morpholine (Tetrahydro-2H-1,4-oxazine)	17.8

1-m column

Parent amine	*Retention time (min)*
Dihexylamine	13.6
N,N-Dimethyl-1,3-propanediamine	64.0
2-Ethoxy-ethylamine	4.8
Dicyclohexylamine	21.0
Dicyclopentylamine	13.4
N-Phenyl-benzylamine	22.6
N,N-Dibenzylamine	36.6
3,4-Xylidine	17.6
2-Naphthylamine	43.6
3-Hydroxypiperidine	10.2
2-Amino-3-picoline	∞
3-(4-Methylamino-butyl)-pyridine	29.6
2-(Methylaminomethyl)-pyridine	10.6
3-(Methylaminomethyl)-pyridine	13.0
4-(Methylaminomethyl)-pyridine	13.0
6-Methyl-2-(methylaminomethyl)-pyridine	11.2
3-(4-Methylamino-1-butenyl)-pyridine	31.6
Nornicotine	23.0
Anabasine	30.4
3-(2-Pyrrolin-2-yl)-pyridine (Myosmine)	20.0

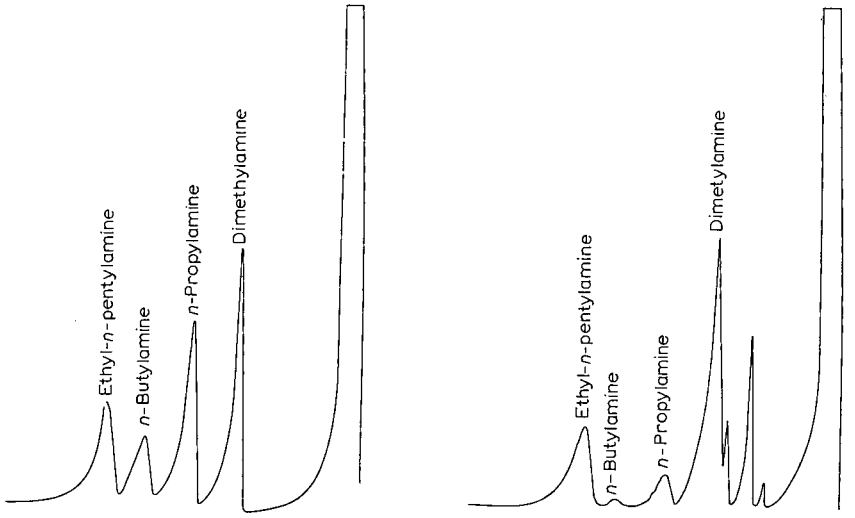

Fig. 7. Mixture of two primary and two secondary amines.

Fig. 8. The same mixture after treatment with nitrous gases for about 40 min.

basic compounds under general analytical conditions. The reaction of the resulting hydrochlorides with 4'-nitroazobenzene-4-carboxylic acid chloride was performed by heating with pyridine. After preseparation on a silica gel column the fractions were separated by thin-layer chromatography. Traces of additional bases in single spots could often be detected by running a gas fractogram thus aiding purification procedures.

Together with other characteristics of the derivatives, *e.g.* melting points, R_F values, elemental analyses, I.R.-spectra, and mass spectra, the retention times are very important for the identification of amines.

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CHROM. 3464

GAS CHROMATOGRAPHIC DETERMINATION OF SUCCINODINITRILE IN PHARMACEUTICALS*

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(Presented December 16th, 1967)

SUMMARY

A gas chromatographic determination of succinodinitrile was worked out which can be used for the analysis of aqueous solutions and mixtures with other therapeutically active substances. The aqueous solution of succinodinitrile is saturated with NaCl, extracted with chloroform, the extract evaporated, dissolved in acetone and chromatographed on silanised Chromosorb W impregnated with diethylene glycol succinate at 205° in a Perkin-Elmer F 11 chromatograph fitted with a flame ionisation detector.

Succinodinitrile is now widely used in medicine to restore cellular nucleoproteins and to re-establish balanced neuropsychological functions. It was therefore thought profitable to investigate the feasibility of a gas chromatographic determination of this substance.

Succinodinitrile has so far been identified by hydrolysing the cyano groups and characterising the resulting succinic acid, while the quantitative analysis has relied on the formation of an insoluble complex with cuprous chloride[1,2] $(CH_2CN)_2Cu_2Cl_2$, or on the conventional determination of nitrogen.

The first report[3] on the separation and identification of nitriles by gas chromatography appeared in 1960, but the results were not always reproducible. Later MUGNAINI AND GAMBELLI[4] reported good separations and quantitative determinations of some nitriles including succinodinitrile. These authors used Apiezon L mixed with sodium caproate as stationary phase on Celite support. They injected 5 μl samples and their chromatograph was equipped with a thermal conductivity detector. In the same year, ARAD-TALMI *et al.*[5] separated a mixture composed of acrylonitrile, propionitrile, butyronitrile, succinonitrile, and adiponitrile in an aqueous acidic solution. The latter was saturated with KCl, extracted with *o*-dichlorobenzene, the extract was dried and then injected into a gas chromatograph in 10–15 μl samples.

The object of this investigation was to devise a gas chromatographic technique for the identification and quantitative determination of succinodinitrile in an aqueous solution, singly or in mixtures with various therapeutically active substances such as

* Translated by Express Translation Service, London S.W. 19.

calcium pantothenate, vitamins B_1, B_2, B_6, B_{12}, and PP, reserpine, dihydroisoandrosterone, benzyl alcohol, sucrose, and natural essences.

In the present method, the sample in an aqueous solution is saturated with NaCl, extracted with chloroform, the extract is concentrated, and the residue is dissolved in acetone and then chromatographed directly. The succinonitrile content is calculated with the aid of the calibration curve shown in Fig. 1, obtained by chromatography of known quantities of succinonitrile processed in the same way.

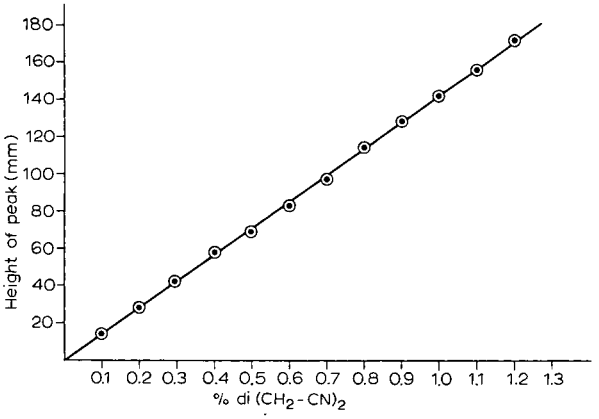

Fig. 1. Chromatographic calibration curve for succinonitrile.

This method is suitable for analysis, in that the standard error is $\pm$ 5 %, which is acceptable in the gas chromatographic determination of quantities of about 3–12 μg. Under experimental conditions, less than 0.1 μg of succinonitrile has been detected, but for ideal reproducibility, the best concentration is 0.1–1.2 mg/ml, since the calibration curve shows a linear relationship in this region.

EXPERIMENTAL

A Perkin-Elmer F 11 chromatograph fitted with a flame ionisation detector (hydrogen flame) was used. The Leeds-Northrup potentiometric recorder (Speedomax G, type S) had a full-scale deflection of 5 mV. The column had a length of 1.60 m and an internal diameter of 1/16 in., and was filled with 80–100 mesh silanised Chromosorb W impregnated with 15 wt. % of diethylene glycol succinate (DEGS). The temperatures of the column and the glass-lined vaporiser were 205 and 300°, respectively. The flow rate of carrier nitrogen was 32 ml/min, the chart speed was 0.5 in./min, and the attenuation factor was 500. Under these conditions the retention time of succinonitrile is 3 min 55 sec.

To construct the calibration curve, aqueous solutions of pure succinonitrile were saturated with NaCl and extracted five times with chloroform. The extracts were evaporated at 80° and the residue was redissolved in acetone, so as to obtain succinonitrile concentrations of 3–12 mg/ml. We then injected 1 μl samples of these solutions, measured the heights of the resulting peaks, and plotted these against the

succinonitrile concentration of the solution. This work was carried out in an isothermal regime.

In the determination of succinonitrile in the presence of other therapeutically active substances, an aqueous solution of the sample was saturated with NaCl and then subjected to the same treatment as described for the calibration samples; the volume of solution was 10 ml and 1 μl samples were injected. The sample was chromatographed under the conditions specified above, and the amount of succinonitrile present in the sample (in %) was determined by measuring the peak height and comparing it with the calibration curve.

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End of papers presented at the
Symposium on the Gas Chromatography of Amine Drugs
Rome, December 15th and 16th, 1967

CHROM. 3660

STUDIES ON THE RING STRUCTURES OF KETOSES BY MEANS OF GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY*

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SUMMARY

Persilylated fructose was separated into five components by means of gas chromatography.

Individual compounds were isolated with the help of micropreparative gas chromatography and characterized by infrared and mass spectroscopy. Components which, because of their small quantity and chemical lability, could not be isolated in this manner were investigated with the instrumental combination of gas chromatography and mass spectroscopy.

Of the five persilylated fructose derivatives two represent the pyranoside, two the furanoside and one the open-chain form.

INTRODUCTION

In a previous study the enzymatic liberation of glucose and fructose from sucrose was investigated using gas chromatography[1]. It was observed that upon persilylation fructose yielded a number of peaks.

In this study it can be shown that after exhaustive persilylation fructose exhibits five components in equilibrium with one another (see Figs. 1a and 2). In addition to these five compounds two incompletely silylated products are observed when a shorter silylation time is used (see Fig. 1b).

Using a gas chromatography–mass spectroscopy combination, the group of peaks 1–3 shown in Fig. 1 resolved into an additional component 3a (see Fig. 2) which appeared after peak 2 and was present in very low concentration.

The individual components were isolated as their trimethylsilyl ether derivatives by means of a micropreparative gas chromatograph, and subsequently characterized, as far as possible, with infrared and mass spectroscopy. Because of their small quantity and ready decomposition, some substances could not be isolated in this manner. These were investigated using an instrumental combination of gas chromatography and mass spectroscopy.

* For a preliminary report see. ref, 15.

** Director: Prof. A. PRADER.

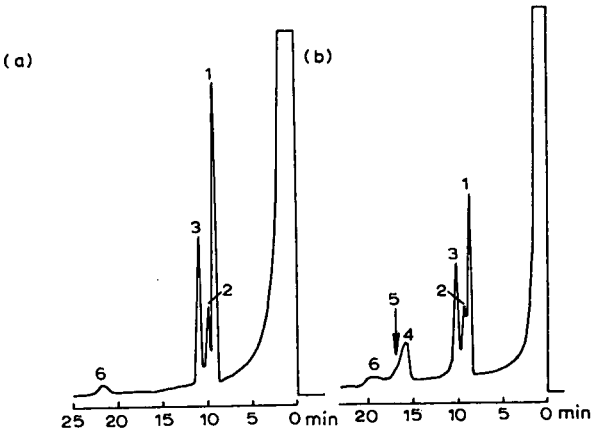

Fig. 1. Gas chromatogram of mutarotated fructose (in water, pH: 5.4) after silylation. (a) 24 h silylation; (b) 1 h silylation. Gas chromatograms were run on an Aerograph "1520" with a split device for preparative work. Column conditions: EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.7 mm; t_c: 145°, t_i: 255°, t_d: 245°; N_2: 45 ml/min.

Enzymatic hydrolysis of sucrose was performed by incubation of sucrose with sucrase–isomaltase complex*, during which samples were removed at different times and frozen in liquid air. This work is fully described in a previous paper[1]. It had already been suspected earlier that the furanoside and pyranoside forms were involved in the mutarotation of fructose according to ISBELL AND PIGMAN[2]. These authors explained the abnormally fast mutarotation of fructose by a pyranose–furanose

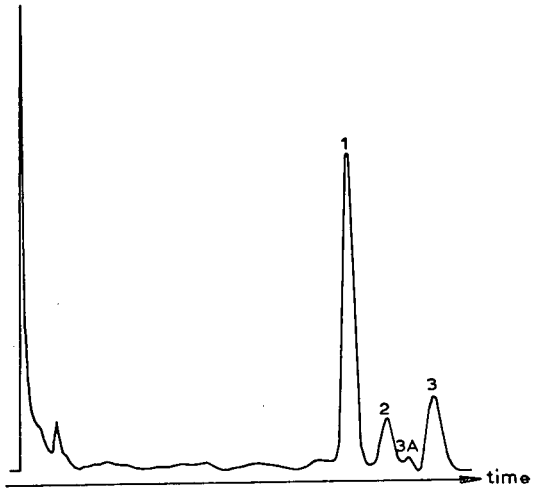

Fig. 2. Gas chromatogram of mutarotated fructose after silylation. Gas chromatograph–mass spectrometer combination: LKB "9000" EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.5 mm; t_c: 150°; t_i: 255°, t_{sep}: 170°; He: 20 ml/min.

* Isolated from rabbit small intestine; courtesy of Prof. G. SEMENZA.

interconversion. GOTTSCHALK[3] was able to show that in aqueous solutions at 0° the equilibrium mixture contained 12% D-fructofuranose. From the corresponding kinetics he calculated that at 20° about 20% of this hexose existed in the furanoside form.

EXPERIMENTAL

Preparation of the samples for the equilibrium mixture

0.1 mg fructose is equilibrated in water for approximately 12 h. The sample is frozen by immersing the incubation tube in liquid air, lyophilized and silylated with 0.1 ml of silylation mixture.

Silylation

The fructose is converted to its O-trimethylsilyl ethers by the method of BENTLEY *et al.*[4] and completely silylated overnight at room temperature.

Preparation of permethylated fructose

The permethylated fructose is synthesized according to the method of KUHN *et al.*[5].

Isolation of methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside (I) and methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside (II) by means of micropreparative gas chromatography

The permethylated fructose is gas chromatographed on an EGS 15% column. The gas chromatogram obtained agrees with the analytical results of BAYER AND WIDDER[6]. The peaks from methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside and from methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside are isolated by means of micropreparative gas chromatography.

Isolation of peaks 1 and 4 as trimethylsilyl ethers from the reaction mixture

Crystalline fructose (10 mg) is dissolved in 20 ml of the silylation mixture and silylated for 1–2 h. Peaks 1, 4 and 5 are almost the only ones formed by this procedure; these are collected, by means of micropreparative gas chromatography, in an absorption tube about 15 cm long and 3 mm wide (I.D.). The procedure consists of injecting 10 μl samples into the gas chromatograph repeatedly until enough of each substance is collected for analysis by infrared and mass spectroscopy. Since peaks 4 and 5 are not completely separable, only the ascending part of peak 4 is collected for analysis. It is not possible to isolate the shoulder, labelled as peak 5, separately from peak 4.

Isolation of peaks 3 and 6 from the reaction mixture

Crystalline fructose (10 mg) is mutarotated for 14 h in water. The aqueous solution is evaporated to dryness under vacuum, the sample dried in a desiccator, and incubated overnight with 5 ml of the silylation mixture. From this reaction mixture, peaks 3 and 6 can be isolated by means of micropreparative gas chromatography and about 100 μg of each substance was collected for analysis by microinfrared and mass spectroscopy. Not enough of peak 2 can be absorbed for analysis and it is identified using a combination of gas chromatography and mass spectroscopy.

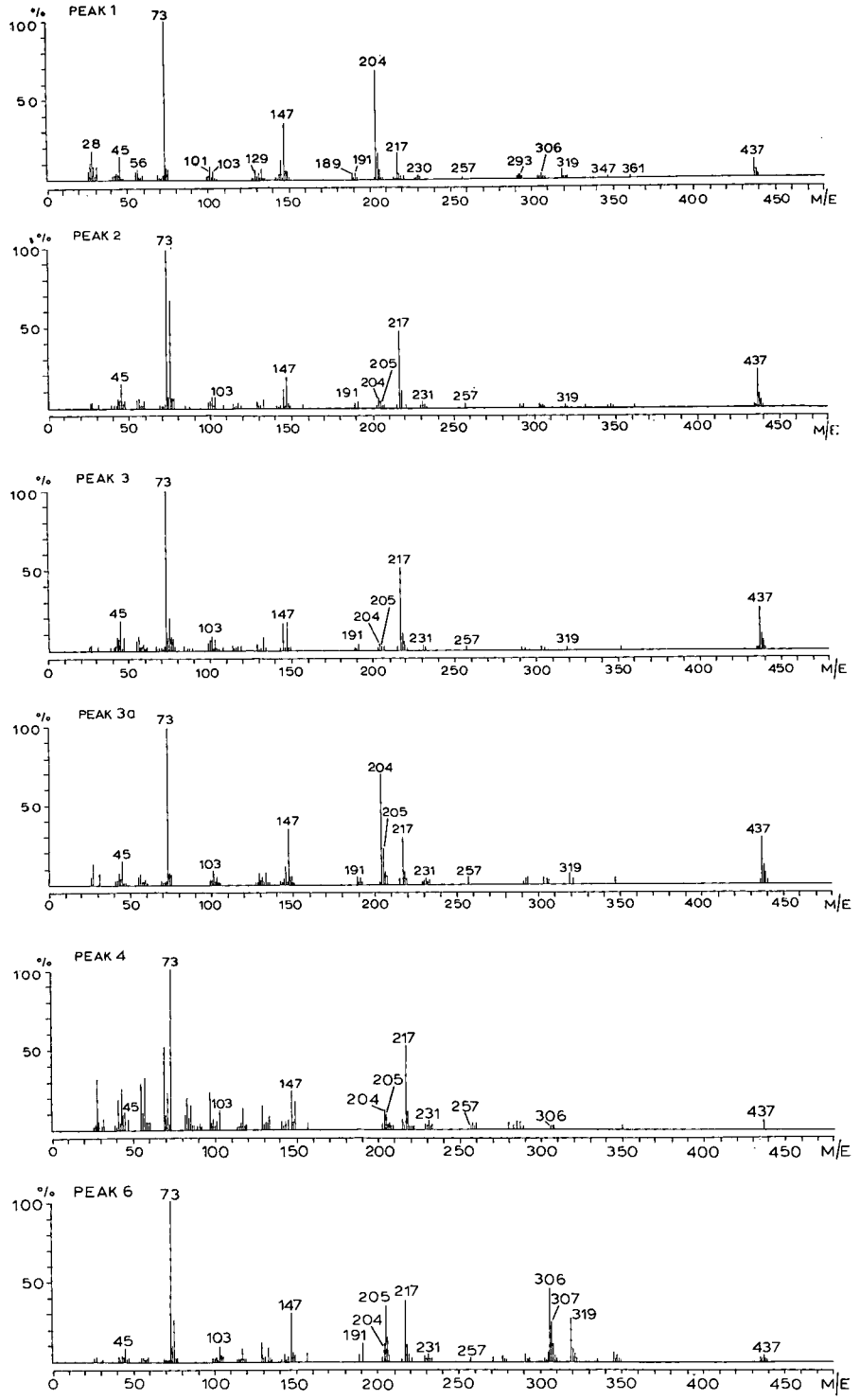

Fig. 3. Mass spectra of the trimethylsilyl ethers of peak 1, 2, 3, 3a, 4, and 6. Peak 1 = M.S.: Hitachi "RMU 6D"; peak 2 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 3 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 3a = G.C./M.S.: LKB "9000"; peak 4 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 6 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D".

RESULTS

The mass spectra of the trimethylsilyl ethers given in Figs. 1 and 2 are shown in Fig. 3. The samples were either collected at the end of the gas chromatographic column and separated, or were led directly into the mass spectrometer. For comparison, two additional mass spectra of permethylated fructose (pyranoside and furanoside forms) are also shown (Fig. 3a).

The mass spectra of the methyl ethers show that the molecular ion ($M^+ = 250$) cannot be detected. Signals of high intensity were *m/e* 219, which could correspond to the cleavage of a methoxy group, *m/e* 88 and 101. It is known from the work of HEYNS *et al.*[7–9] and KOCHETKOV AND CHIZHOV[10] that the major difference between the mass spectra of the pyranoside and furanoside forms of permethylated pentoses and hexoses consists in the relative intensities of the signals at *m/e* 88 $(CH_3O—CH=CH—OCH_3)^+$ and *m/e* 101 $(CH_3O=CHCH=CHOCH_3)^{+*}$. The mass spectrum of the pyranosides was characterized by a very strong signal at *m/e* 88 (the second strongest, 98 %)

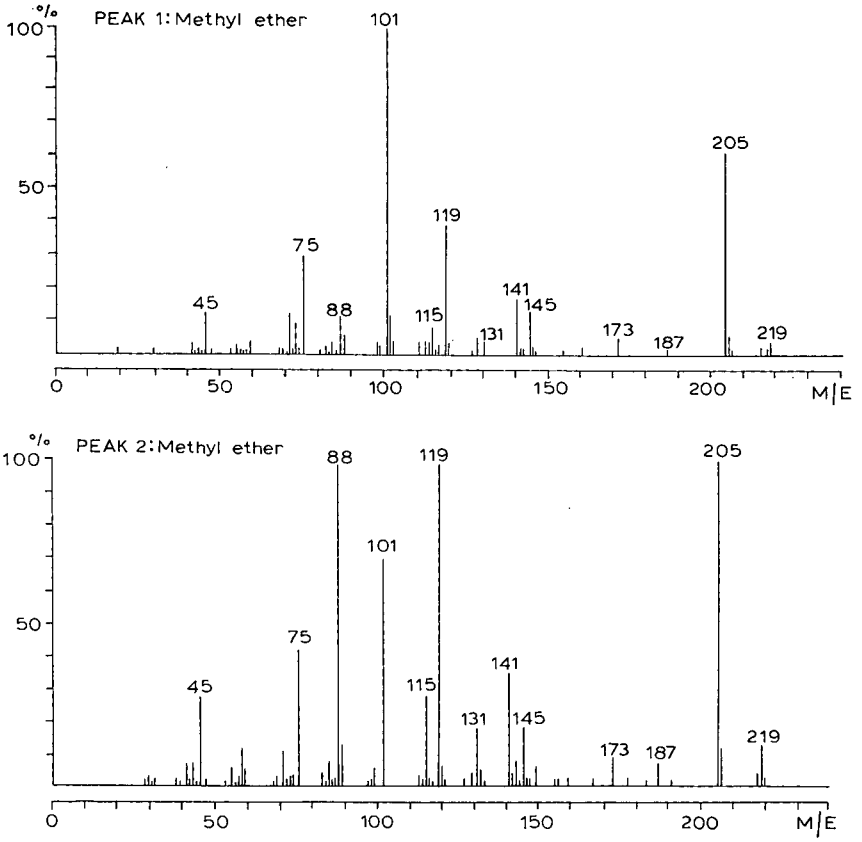

Fig. 3a. Mass spectra of the methyl ethers of methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside (I) and methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside (II). M.S.: Varian "M 66".

* For explanation of this and other formulations of mass spectral fragments used in this paper see H. BUDZIKIEWICZ, D. DJERASSI AND D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, Holden-Day Inc., San Francisco, 1967.

and by a signal at *m/e* 101 amounting to 69%. The spectrum of the furanosides, on the contrary, was characterized by the almost complete lack of the signal at *m/e* 88 (less than 13%) and by the signal at *m/e* 101 amounting to 100%.

Similar behavior was exhibited by the trimethylsilyl ethers, as also noted by HEYNS *et al.** The molecular ion ($M^+ = 540$) was not observable. The highest mass number was at *m/e* 437 which could be attributed to the loss of a $-CH_2OSi(CH_3)_3$ group. Intense signals were always found at either *m/e* 204 or 217 (peaks 1, 2, 3, and 3a). These signals correspond to the mass numbers at *m/e* 88 and 101 respectively, observed for the methyl ethers. It was striking that the mass spectra of chromatographic peaks 1 and 3a on the one hand, and those of 2 and 3 on the other, were practically identical. For the former pair, the mass at *m/e* 204 was very intense, while that at *m/e* 217 was weaker. For the latter, the *m/e* 204 signal was almost non-existent, whereas a very strong signal was exhibited at *m/e* 217.

In analogy to the mass spectra of the methyl ethers, the peaks 1 and 3a should be attributed to the pyranoside forms (β- and α-anomers). This assignment was in agreement with the work of HEYNS *et al.*[7–9] and of KOCHETKOV AND CHIZHOV[10], who established that high abundance of mass number *m/e* 88 in methyl ethers indicated a pyranoside form.

Further confirmation of the correctness of the foregoing correlation arises from the following facts:

1. Crystalline fructose exists in the β-pyranoside form[11]. Gas chromatography of persilylated fructose resulted in practically only peak 1. Thus, this peak is very likely to correspond to the β-pyranoside form.

2. In sucrose, fructose exists in the furanoside form. When sucrose was split by intestinal sucrase, which is an α-glucosido-hydrolase, two major peaks of fructose were produced (3 and 1). The peak 1:peak 3 ratio increased as the hydrolysis proceeded. On extrapolation to zero time, only peak 3 was present in the mixture. This indicates that peak 3 is a furanoside.

Fig. 4 shows gas chromatograms of the monosaccharides which were formed during the enzymatic cleavage of sucrose with sucrase at various incubation periods[1].

The results of the cleavage studies and the ratio of the peak areas (peak 1:peak 3) are given together in Table 1.

3. It is known that, generally, the form which contains the least substituents in the axial position is the most stable. From this it would follow that component 1 is in the β-form. This would also explain the vanishingly small amount of component 3a. For the quasi-planar 5 membered ring form, no unequivocal distinction was possible between the axial and equatorial substituents. It would be a reasonable guess to ascribe peak 3 to the β-furanose since this is the initial product of the enzymatic hydrolysis of sucrose. Peak 2 is then the α-furanose.

Components 4 and 5 appeared to be incompletely silylated products. In contrast to components 1 and 3 which did not show an –OH band in the infrared spectrum, components 4 and 5 did have a band at 3460 cm^{-1}**.

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* Personal communication of D. MÜLLER, Chemical Institute, Department of Organic Chemistry, University of Hamburg.

** I.R.: A Perkin-Elmer "257" with a beam condensor and micro-sodium chloride cuvettes with a 2 μl capacity was used. The infrared spectra of the trimethylsilyl ethers were taken in carbon tetrachloride.

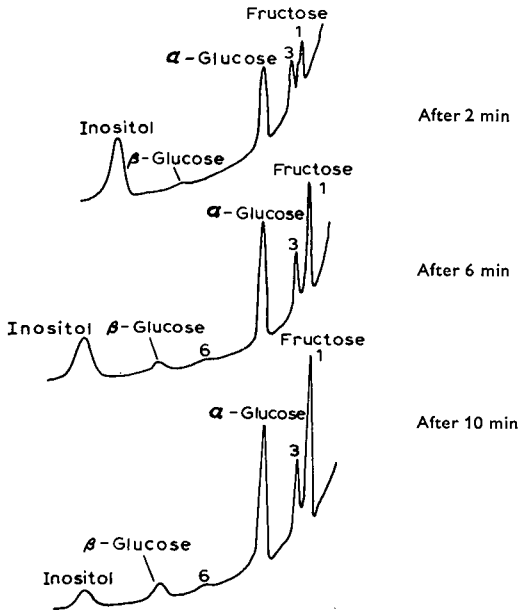


Fig. 4. Gas chromatograms of the monosaccharides formed during enzymatic cleavage after 2, 6, and 10 min. G.C.: Aerograph "1520" with split device for preparative work. EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.7 mm; t_c: 145°, t_i: 255°, t_d: 245°; N_2: 45 ml/min.

The mass spectrum of component 4 also showed a mass number at 437 indicating that not more than one trimethylsilyl group was missing.

When longer silylation times were employed (24 h), components 4 and 5 disappeared while peak 1 increased. This phenomenon is easily explained by the hypothesis of an incomplete silylation.

TABLE I

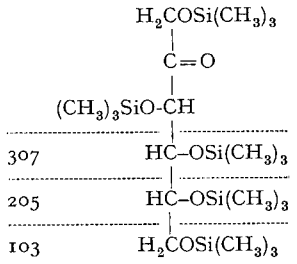
MONOSACCHARIDES: LIBERATED DURING THE HYDROLYSIS OF SUCROSE WITH INTESTINAL SUCRASE

Sample	*Time*	*Glucose (μg)*			*Fructose (μg)*				
		α	*β*	*Total*	*1*	*3*	*1/3*	*6*	*Total*
A	2 min	33.3	3.7	37	15.5	17.5	0.89	—	33
B	6 min	85.1	12.9	98	36.7	31.3	1.17	2.0	70
C	10 min	265.0	44.0	309	194.5	108.6	1.79	7.9	311
D	20 min	283.8	114.2	398	306.8	137.6	2.23	11.6	456

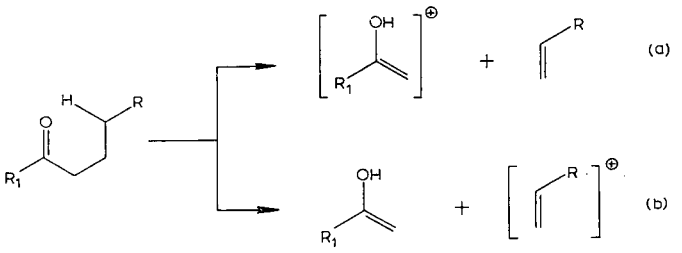
In contrast to the remaining compounds, component 6 showed a keto band at 1735 cm^{-1} in the infrared spectrum. No –OH band was present. This pointed to an open-chain form. The mass spectrum showed a decomposition pattern different from

that of the ring forms. The peak of highest mass number was, once again, 437. Intense signals could be found at *m/e* 103, 205, and 217, while new mass numbers appeared at *m/e* 306, 307, and 319.

The odd mass numbers *m/e* 103, 205, and 307 can be explained through simple cleavage:



The intense fragment of mass number *m/e* 306 must arise from a hydrogen rearrangement. One possibility is the McLAFFERTY rearrangement[12], represented as:



It is known[13] that not both possibilities of charge distribution will necessarily occur; it is rather the stability of the resulting ion as well as of the unchanged fragment which is determinative for the fragmentation pattern.

In the present case, practically only reaction (b) would occur.

DISCUSSION

From the foregoing results it is seen that, of the five persilylated fructose derivatives two are the pyranoside, two the furanoside and one the open-chain form of fructose. This conclusion is drawn from M.S., I.R. and gas chromatographic studies. We find in an equilibrium mixture of fructose about 33 % furanoside and about 67 % pyranoside, corresponding closely to the observations of GOTTSCHALK.[3]

The free, open-chain form of fructose was isolated as its trimethylsilyl ether derivative from aqueous solution at equilibrium. It was judged to be chromatographically pure and the structure was assigned with the aid of I.R. and M.S. spectra. However, this does not unconditionally prove the actual presence of the open-chain form in aqueous fructose solutions at room temperature.

The isolation of a methylated fructose in the keto form was described by BAYER AND WIDDER[14] in 1965; 60 % was the concentration (purity) of the isolated compound. However, the use of more drastic reaction conditions in BAYER's methyl ether

formation may have introduced a bias in the composition of the original equilibrium mixture.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Prof. W. SIMON and P. D. J. SEIBL of the Swiss Federal Institute of Technology, Zürich as well as to P. D. H. BUDZIKIEWICZ, Institute of Technology, Braunschweig, for providing mass spectra and for valuable advice.

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CHROM. 3632

GELCHROMATOGRAPHISCHE TRENNUNG DER NUCLEINSÄUREBAUSTEINE AN SEPHADEX G-25 FINE, G-15 UND G-10

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SUMMARY

Gel chromatographic separation of nucleic acid components on Sephadex G-25 fine, G-15 and G-10

After a brief reference to publications which deal almost exclusively with qualitative separations of nucleic acid components on Dextran gels, elution diagrams and tables are used to show which factors are responsible for the quantitative separation of complicated mixtures. The lower as well as narrower fractionation range of dextran gels G-15 and G-10 at a bed volume 1.3 × 150 cm permits the separation of molecules which differ in their chemical molecular or steric structure.

In addition the drop rate as well as the pH value and the ionic strength of the eluent influence the elution more or less strongly retardable purine and pyrimidine bases and their sugar derivatives.

Separations carried out in the μg range, show that in quantitative evaluation of the elution diagrams the losses are only minimal. Thus this type of gel chromatography is not only a rapid but also an accurate method for the quantitative estimation of very small amounts of components of nucleosides present in nucleic acid hydrolysates.

Schon PORATH[1] und GELOTTE[2] beobachteten bei niedermolekularen Verbindungen eine zum Teil recht unterschiedliche Retardierung derselben an der Gelmatrix, die in der Gelchromatographie als "reversible Adsorption" bezeichnet wird. GELOTTE[2] bestimmte eine grosse Zahl von K_d-Werten solcher niedermolekularer aromatischer und heterocyclischer Verbindungen und deutete die Erscheinungen mit den Wechselwirkungen zwischen gelöstem Stoff und Gelgerüst. Dieselben Beobachtungen konnten ZADRAZIL, SORMOVA UND SORM[3] sowie HOHN UND POLLMANN[4] bei der Trennung von Nucleinsäurebausteinen an Sephadex G-25, machen; ein Gel mit relativ hohem Fraktionierungsbereich im Vergleich zum Molekulargewicht dieser Verbindungen. Weiters erschien im Novemberheft 1967 dieser Zeitschrift ein Beitrag von DE BERSAQUES[5] über Trennung von Nucleinsäurebausteinen an den stärker quervernetzten Sephadex-Typen G-25 superfine, G-15 und G-10. Nach eigenen Erfahrungen kann an der von DE BERSAQUES eingesetzten Säule (1,5 × 25 cm) nur eine

qualitative Trennung sehr unterschiedlich retardierbarer Purin- bzw. Pyrimidinderivate erreicht werden.

METHODIK

Mit einem der in dest. Wasser gequollenen Dextrangele G-25 fine, G-15 oder G-10 wurde ein Chromatographierrohr mit den Abmessungen 1.3 × 153 cm bis zu einer Höhe von 150 cm gefüllt und mit 200 ml der Elutionsflüssigkeit (0.01 *M* Ammoniumkarbonatlösung[4]) äquilibriert. Die Probelösungen (nur Gemische der reinen Substanzen) betrugen, bei einer Konzentration von 1–2 mg/ml und Substanz, 150 μl. Die Elutionsflüssigkeit wurde mittels Schlauchpumpe der Säule zugeführt und das Eluat in 5 ml Fraktionen aufgefangen. Die Eluate wurden bei den für die einzelnen Bausteine charakteristischen Absorptionsmaxima im U.V.-Bereich gemessen (PMQ II von Zeiss; Spalt 0.15 und 2 cm Quarzküvetten).

ERGEBNISSE UND DEREN DISKUSSION

In den folgenden Versuchen soll die Möglichkeit der quantitativen Trennung eines komplexen Gemisches von Nucleinsäurebausteinen an den Gelen G-15 und G-10 aufgezeigt werden. Für den ersten Versuch wurde das gleiche Gemisch unter gleichen Bedingungen an G-25 fine und G-15 chromatographiert (Fig. 1 und 3). Der relativ

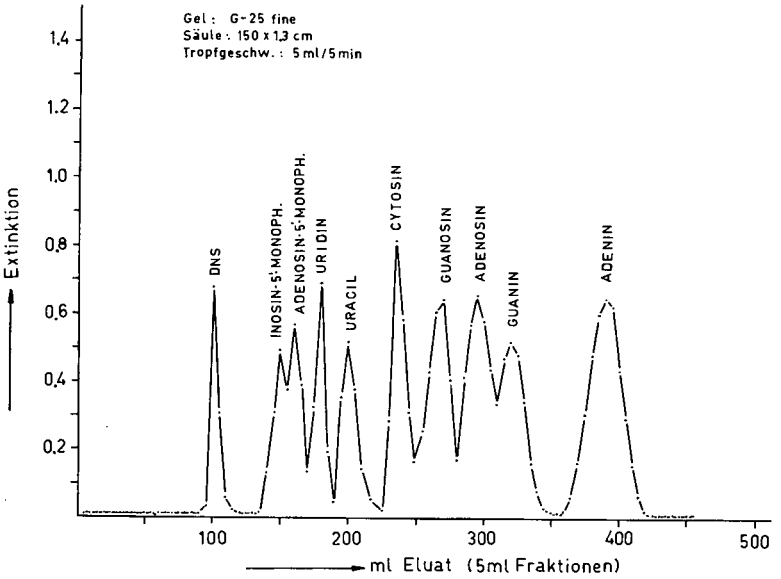

Fig. 1. Fraktogramm eines komplexen Gemisches von Nucleinsäurebausteinen, getrennt an G-25 fine.

weite und hohe Fraktionierungsbereich des G-25 fine erlaubt nur teilweise eine quantitative Trennung. Die Wechselwirkungen zwischen gelöstem Stoff und Gelmatrix sind bei dem relativ weitporigen Gel noch nicht so ausgeprägt wie bei den viel stärker quervernetzten Typen G-15 und G-10. An der G-15-Säule wurde das gleiche

Gemisch, bis auf Adenosin–Guanin, quantitativ aufgetrennt. Dass es sich hier nicht um eine reine Gelfiltration handelt, beweisen die viel höheren Elutionsvolumina der Purinbasen gegenüber den Pyrimidinbasen mit niederem Molekulargewicht. Diese Tatsache macht sich noch bei den Nucleotiden derselben (Adenosin-5'-monophosphat

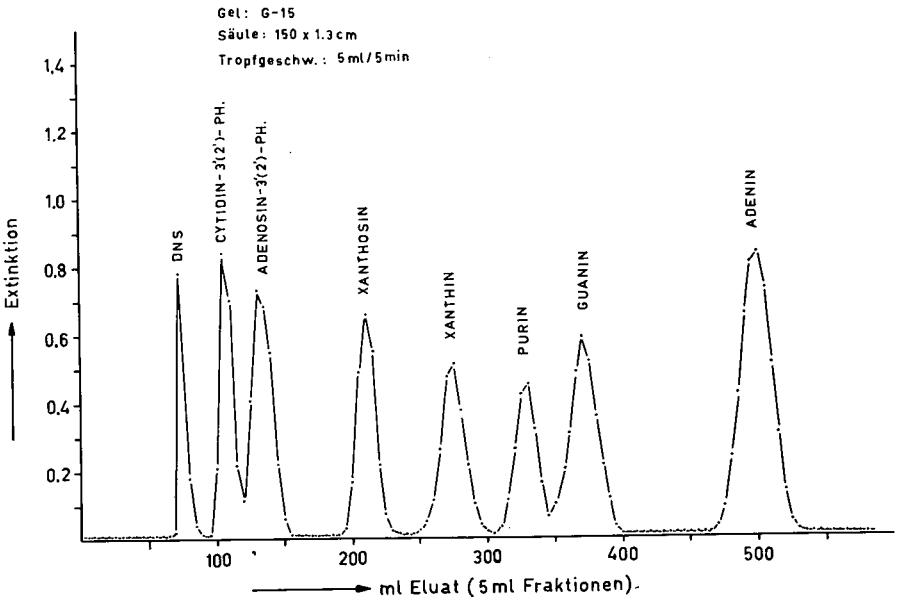

Fig. 2. Fraktogramm eines Gemisches verschiedener Purinbasen. Unterschiedlicher Einfluss der NH_2- und phenolischer O-Gruppen auf die Retardierbarkeit des Moleküls.

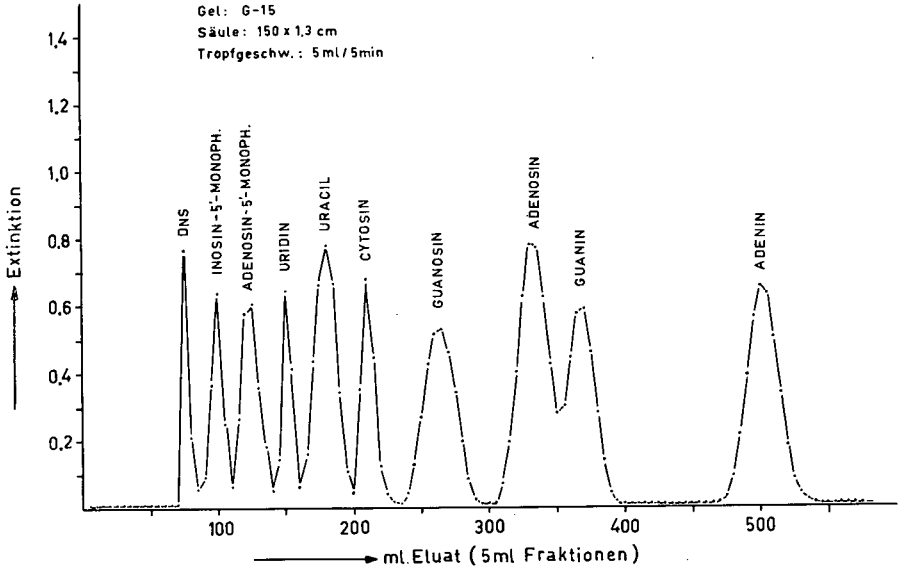

Fig. 3. Fraktogramm des gleiches Gemisches wie in Fig. 1, diesmal an G-15 aufgetrennt. Der engere Fraktionierungsbereich des G-15 ist deutlich zu erkennen.

und Cytidin-3'(2')-phosphat, Fig. 2 und 3) deutlich bemerkbar; eine qualitative Trennung der beiden Nucleotide ist durchaus möglich. Abgesehen von der unterschiedlichen Retardierbarkeit der Purin- und Pyrimidinabkömmlinge sind spezielle Merkmale der Moleküle untereinander von grosser Bedeutung für eine quantitative Trennung bei nur geringfügigem Unterschied in den Molekulargewichten. Am deutlichsten ist der Einfluss der Aminogruppe im Molekül (Fig. 2). 6-Aminopurin (Adenin), das nur um die NH_2-Gruppe gegenüber Purin "schwerer" geworden ist, wird um 34 Fraktionen (= 170 ml) später aus der Säule eluiert. Der Ersatz der Aminogruppe durch eine phenolische (Hypoxanthin) setzt die Retardierbarkeit des Moleküls deutlich herab (Fig. 4). Das gleichzeitige Vorhandensein einer Amino- und einer phenolischen Gruppe (2-Amino-hydroxypurin = Guanin) bestätigt den grösseren Einfluss des ersteren auf das Elutionsverhalten des Moleküls (Fig. 2). Noch deutlicher wird dies beim Vergleich der Elutionsvolumina von Uracil (2,6-Dihydroxy-pyrimidin) und Cytosin (2-Hydroxy-

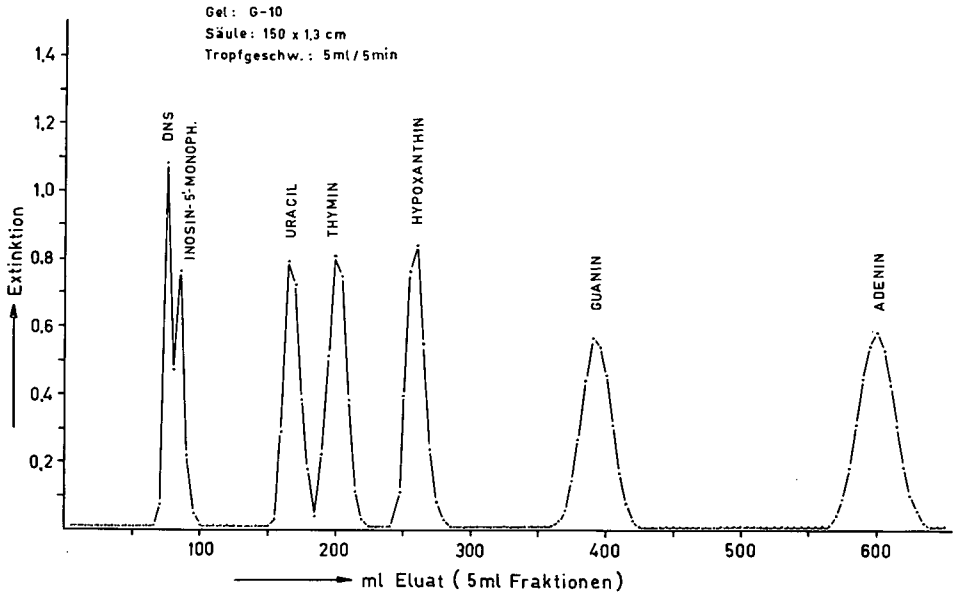

Fig. 4. Trennung des Basenpaares Uracil–Thymin and Gel G-10.

6-amino-pyrimidin) in Fig. 3. Im Molekulargewicht ist praktisch kein Unterschied vorhanden (Tabelle III) und dennoch ist eine quantitative Trennung der beiden Pyrimidinbasen an einer Säule mit G-15 möglich.

Neben diesem chemisch-molekularen Aufbau der Moleküle können auch solche sterischer Natur für eine unterschiedliche Aufenthaltsdauer des Moleküls in einem Dextrangelbett verantwortlich sein. Diese Beobachtung konnte beim Basenpaar Uracil–Thymin gemacht werden (Fig. 4). Bei einer Tropfgeschwindigkeit von ml/Min. oder langsamer sind die beiden Pyrimidinderivate an G-10 quantitativ voneinander zu trennen, woran vermutlich der räumliche Aufbau der Moleküle nicht unwesentlich beteiligt ist, denn der Unterschied im Molekulargewicht ist sehr gering. Der beachtliche Unterschied im Elutionsverhalten (Separationsvolumen = 35 ml) ist somit auf eine Diffusionsbehinderung des Thymins zurückzuführen. In der schematisierten

Wiedergabe der Elutionsvolumina der Nucleinsäurebausteine in Fig. 5 fällt auf, dass beim Übergang zu den stärker quervernetzten Dextrangelen die Basen Adenin und Guanin sowie das Nucleosid Adenosin viel stärker retardiert werden als die anderen Basen und Nucleoside; letztere erscheinen mit zunehmenden Vernetzungsgrad des

TABELLE I

PROZENTUELLE VERLUSTE DER AN G-15 CHROMATOGRAPHIERTEN NUCLEINSÄUREBAUSTEINE

Nucleinsäurebausteine	*Aufgetragene Menge (µg)*	*Prozentuelle Verluste (Mittelwert von 6 Bestimmungen)*
Adenin	150	−3.15 %
Adenosin	150	−2.95 %
Adenosin-5′-monophosphat	150	−2.70 %
Guanin	150	−2.25 %
Guanosin	150	−0.70 %
Guanosin-5′-monophosphat	150	−1.70 %
Cytosin	150	−2.60 %
Cytidin	150	−2.35 %
Cytidin-3′(2′)-phosphat	150	−2.40 %
Uracil	150	−2.50 %
Uridin	150	−2.00 %
Uridin-5′-monophosphat	150	−2.15 %
Thymin	150	−2.85 %
Thymidin	150	−2.20 %
Thymidin-5′-monophosphat	150	−2.00 %

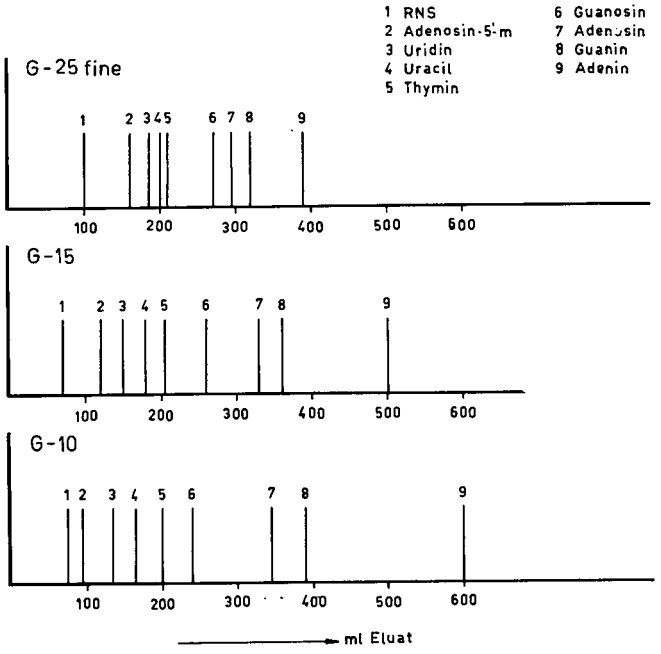

Fig. 5. Schematische Wiedergabe der Peakelutionsvolumina. Einfluss des Vernetzungsgrades der Gele auf das Elutionsverhalten der Nucleinsäurebausteine.

Dextrangels immer früher im Eluat. Das bedeutet aber, dass sämtliche Pyrimidinbasen, deren Nucleoside sowie die Purin- und Pyrimidinnucleotide samt Guanosin beim Übergang zu G-15 und G-10 mehr und mehr ausgeschlossen werden bzw. nimmt ihre Retardierbarkeit immer stärker ab.

Einfluss der Tropfgeschwindigkeit auf die Trennung

Nicht unwesentlich ist die Tropfgeschwindigkeit des Elutionsmittels gerade bei diesen zum Teil recht beachtlich retardierbaren Verbindungen. Eine Erhöhung der Tropfgeschwindigkeit von 5 ml/5 Min. auf 5 ml/1 Min. 30 Sec. hat nur auf die stärker adsorbierten stickstoffhaltigen Heterocyclen wie Adenin, Guanin, Adenosin, Guanosin und Adenosin-5'-monophosphat einen Einfluss; diese Purinderivate werden bei der erhöhten Tropfgeschwindigkeit zum Teil wesentlich früher aus der Säule eluiert, womit aber die quantitative Trennung ein und desselben Gemisches nicht beeinträchtigt wird. So wird Adenin bei sonst gleichen Bedingungen um 24 Fraktionen (120 ml), Guanin um 5, Adenosin um 4, Guanosin um 2 und Adenosin-5'-monophosphat um 2 Fraktionen früher im Eluat erscheinen. Alle anderen in den Diagrammen bzw. der Tabelle III angeführten Purin- und Pyrimidinderivate weisen bei der erhöhten Tropfgeschwindigkeit ein unverändertes Elutionsverhalten auf. Wesentlich ist die Tropfgeschwindigkeit – wie schon oben erwähnt – bei der quantitativen Trennung der Pyrimidinbasen Uracil und Thymin. Ob eine Tropfgeschwindigkeit des Elutionsmittels von 5 ml/5 Min. oder 5 ml/1 Min. 30 Sec. gewählt wird, die Peakelutionsvolumina (= Elutionsvolumen, bei welchem die Substanz im Eluat in maximaler Konzentration erscheint) der beiden Pyrimidinderivate bleiben unverändert, nur ist bei der höheren Tropfgeschwindigkeit keine quantitative Trennung möglich.

Einfluss des pH-Wertes auf die Trennung

Sowohl pH-Wert wie Ionenstärke des Elutionsmittels beeinflussen die Retardierbarkeit der Nucleinsäurebausteine (Fig. 6). So beobachtet man beim Übergang von 0.01 *M* zu 0.03 *M* $(NH_4)_2CO_3$-Lösung eine erhöhte Adsorption für Adenin und Guanin und eine gleichzeitige Zunahme des Separationsvolumens. Die Elutionsbanden werden breiter; die Einstellung des Adsorptionsgleichgewichtes ist sichtlich gestört. In diesem Fall wurde der pH-Wert nur um 0.5 erhöht, aber um so mehr die Ionenstärke des Elutionsmittels, woraus die erhöhte Retardierbarkeit der Purinderivate resultiert. 0.01 *N* HCl und dest. Wasser vermindern die Adsorptionsfähigkeit sämtlicher Purin- und Pyrimidinderivate erheblich, wobei die 0.01 *N* HCl nur eine qualitative Trennung der Basen Adenin und Guanin gestattet, wogegen dest. Wasser gerade noch eine quantitative Auftrennung des Basenpaares an einer G-10 Säule ermöglicht. Ähnlich verhalten sich die Zuckerderivate dieser Purinbasen (Fig. 6). Lediglich beim Übergang zur 0.03 *M* Ammoniumkarbonatlösung werden sie etwas schwächer adsorbiert, womit die quantitative Trennung des Guanins von Adenosin erst möglich ist.

Quantitative Auswertung der Elutionsdiagramme

Auf Grund der relativ starken Retardierbarkeit einiger Nucleinsäurebausteine stellt sich die berechtigte Frage, ob die Elution dieser Verbindungen auch quantitativ erfolgt. Für die Auswertung solcher Fraktogramme gibt es bekanntlich mehrere Methoden. In der vorliegenden Arbeit wurden die einzelnen Punkte der Elutions-

TABELLE II

PROZENTUELLE VERLUSTE DER AN G-10 CHROMATOGRAPHIERTEN NUCLEINSÄUREBAUSTEINE

Nucleinsäurebausteine	*Aufgetragene Menge* (*μg*)	*Prozentuelle Verluste* (*Mittelwert von 6 Bestimmungen*)
Adenin	150	−3.50%
Adenosin	150	−2.20%
Adenosin-5′-monophosphate	150	−3.25%
Guanin	150	−2.40%
Guanosin	150	−2.00%
Guanosin-5′-monophosphat	150	−2.35%
Cytosin	150	−2.90%
Cytidin	150	−2.25%
Cytidin-3′(2′)-phosphat	150	−2.00%
Uracil	150	−2.35%
Uridin	150	−2.20%
Uridin-5′-monophosphat	150	−2.25%
Thymin	150	−2.35%
Thymidin	150	−1.90%
Thymidin-5′-monophosphat	150	−1.85%

TABELLE III

K_d UND K_{av}-WERTE DER NUCLEINSÄUREBAUSTEINE

Elutionsmittel: 0.01 *M* $(NH_4)_2CO_3$-Lösung

Nucleinsäurebausteine	*G-25 fine*		*G-15*		*G-10*		*MG*
	K_{av}	K_d	K_{av}	K_d	K_{av}	K_d	
Adenin	2.90	3.51	3.30	4.62	4.20	6.56	135
Adenosin	1.95	2.15	2.00	2.79	2.16	3.37	267
Adenosin-5′-monophosphate	0.60	0.80	0.42	0.58	0.16	0.25	347
Guanin	2.20	2.70	2.30	3.22	2.52	3.93	151
Guanosin	1.70	1.90	1.50	2.09	1.36	2.21	283
Guanosin-5′-monophosphate	0.60	0.80	0.30	0.43	0.12	0.18	363
Uracil	1.00	1.20	0.84	1.18	0.72	1.12	112
Uridin	0.80	1.00	0.61	0.85	0.48	0.75	244
Uridin-5′-monophosphate	0.50	0.70	0.07	0.10	0.08	0.12	324
Cytosin	1.35	1.55	1.07	1.50	0.96	1.50	111
Cytidin	1.05	1.25	0.84	1.18	0.68	1.06	243
Cytidin-3′(2′)-phosphate	0.55	0.75	0.27	0.37	0.08	0.12	323
Thymin	1.10	1.30	1.03	1.45	1.00	1.56	126
Thymidin	0.90	1.10	0.73	1.02	0.48	0.75	242
Thymidin-5′-monophosphate	0.55	0.75	0.18	0.26	0.20	0.31	322
Purin	—	—	2.00	2.79	2.16	3.37	120
Xanthin	—	—	1.57	2.20	1.44	2.31	152
Xanthosin	—	—	1.07	1.50	0.84	1.94	284
Hypoxanthin	—	—	1.57	2.20	1.48	2.31	136
Inosin	—	—	1.10	1.50	0.85	1.95	268
5-Methylcytosin	—	—	1.10	1.60	1.00	1.56	125
RNS	0.00	0.00	0.00	0.00	0.00	0.00	—
DNS	0.00	0.00	0.00	0.00	0.00	0.00	—

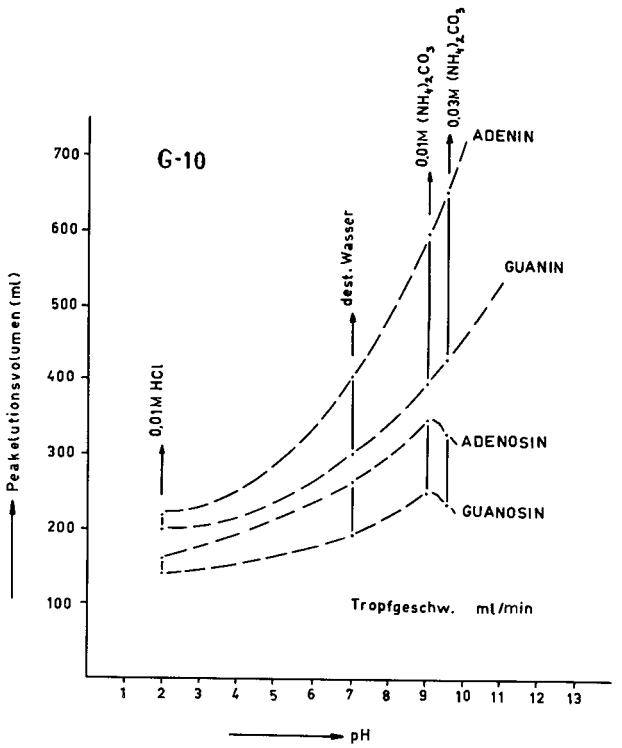

Fig. 6. Änderung des Peakelutionsvolumens durch den pH-Wert sowie Ionenstärke des Elutionsmittels.

banden mit denen einer entsprechenden Eichgeraden verglichen und daraus die Gesamtmenge des eluierten Bausteines ermittelt. Die in den Tabellen I und II angeführten Mittelwerte (Verluste in %) stellen das Mittel aus 6 Einzelwerten dar, wobei die Streuungen um den Mittelwert mit rund ± 0.5 % angegeben werden können.

Damit ein Vergleich mit anderen Arbeiten möglich ist, bedarf es der Angabe von K_{av} – bzw. K_d – Werten. Die Unsicherheit der Methoden zur Bestimmung des "inneren" Volumens V_i bedeutet, dass andere Parameters gesucht werden müssen um das Elutionsverhalten der Substanzen zu beschreiben. Geeigneter erscheint es den K_{av}-Wert ($K_{av} = V_e - V_o / V_t - V_o$) nach LAURENT UND KILLANDER[6] anzugeben, ein Wert in dem das "innere" Volumen V_i nicht berücksichtigt wird. Die in der Formel aufscheinenden Grössen (Volumina) sind wesentlich einfacher zu messen. In der Tabelle III sind zum Vergleich beide Konstanten angeführt.

ZUSAMMENFASSUNG

Nach einem kurzen Hinweis auf Veröffentlichungen, die fast ausschliesslich qualitative Trennungen der Nucleinsäurebausteine an den Dextrangelen beinhalten, wird an Hand von Elutionsdiagrammen und Tabellen demonstriert welche Faktoren für eine quantitative Trennung eines komplizierteren Gemisches verantwortlich sind. Der niedere sowie engere Fraktionierungsbereich der Dextrangele G-15 und G-10 bei

einem Gelbettvolumen von 1.3 × 150 cm, erlauben es Trennungen von Molekülen vorzunehmen deren Unterschiede im chemisch-molekularen wie im sterischen Aufbau liegen. Auch die Tropfgeschwindigkeit sowie der pH-Wert und die Ionenstärke des Elutionsmittels beeinflussen das Elutionsverhalten der mehr oder minder stark retardierbaren Purin- und Pyrimidinbasen bzw. deren Zuckerderivate. Die im μg-Bereich durchgeführten Trennungen lassen erkennen, dass bei einer quantitativen Auswertung der Elutionsdiagramme nur mit minimalen Verlusten zu rechnen ist. Somit ist diese Art der Gelchromatographie nicht nur eine schnelle sondern auch eine recht genaue analytische Methode um noch kleinste Mengen der in einem Nucleinsäurehydrolysat vorkommenden Basen bzw. Nucleoside auch quantitativ zu erfassen.

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CHROM. 3533

A MATHEMATICAL TREATMENT OF THE VARIATION OF THE R_F VALUE CAUSED BY THE PRESENCE OF ANOTHER COMPONENT

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SUMMARY

The R_F value of a component (A) of a mixture is influenced by the presence of another component (B) if the latter alters the distribution coefficient, α_A. The results obtained show that ΔR_M (the difference between the R_M values in the presence and absence of the other component) is directly proportional to the amount of the other component and can be given by the equation:

$$\Delta R_M = K.m_B$$

where K is a constant and m_B is the amount of component B.

This rule can be used to obtain real $R_{F(0)}$ values, by determining $R_{M(i)}$ values for component A in the presence of various amounts of component B and then extrapolating to zero.

The presence of other components in a mixture may have a great effect on the reproducibility of R_F values for various substances. It is, however, impossible to anticipate this influence. The mathematical treatment of this problem is interesting and it can help in chromatographic work since by mathematical extrapolation it is possible to obtain the correct R_F value.

PRINCIPLE

According to the equation of MARTIN AND SYNGE[1], the R_F value is a function of the distribution coefficient α ($\alpha = C_S/C_M$) and the ratio of the quantity of the cross sectional areas occupied by the mobile and stationary phases (A_M/A_S). In the ideal case the following relation holds:

$$\frac{1}{R_F} = \frac{A_M}{A_S} \cdot \alpha + 1 \qquad (1)$$

The purpose of this work was to study the change in the R_F value caused by the

presence of another component. The functional dependence of ΔR_F may be given by the equation:

$$\Delta R_F = F\left[\Delta a, \Delta\left(\frac{A_M}{A_S}\right), \Delta d_s\right] \tag{2}$$

where

$\Delta\alpha$ = the change in α,
$\Delta(A_M/A_S)$ = the change in the ratio A_M/A_S,
d_s = the variation of the layer thickness.

Supposing that $\Delta\,(A_M/A_S) \to 0$ and $\Delta d_s \to 0$, eqn. (2) becomes:

$$\Delta R_{F(A)} = f(\Delta a_A) \tag{3}$$

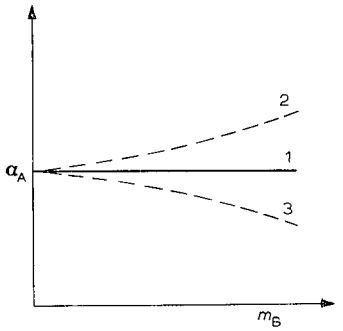

Fig. 1. The influence of component B on α_A. (1) Component B does not effect α_A; (2) component B causes an increase in α_A; (3) component B causes a decrease in α_A.

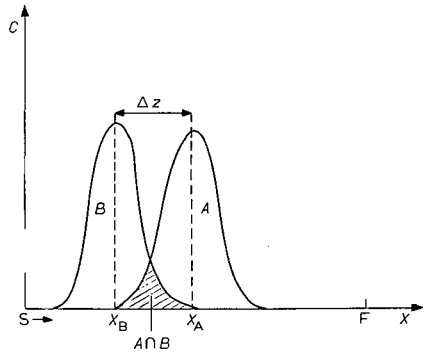

Fig. 2. Diagram showing the ratio of the amount of component B that influences component A ($A \cap B/A$).

The distribution coefficient α_A for a substance A depends on the chromatographic system. The presence of another component, B, changes the properties of the system and thus can also change α_A, as is shown in Fig. 1. Analogously component A can change α_B.

At the start the influence of component B on α_A is greatest, but as the chromato-

gram develops this influence dimishes because of the increasing distance between the components.

Theoretically, four cases are possible, *viz.*:

(1) If component B (low R_F value) influence component A (high R_F value) so that α_A increases, the time of overlapping of the components during the chromatographic process is prolonged.

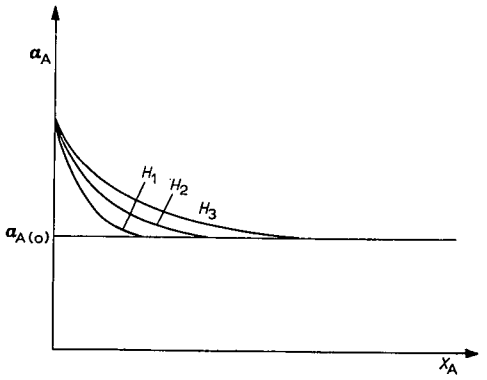

Fig. 3. The change in α_A during the chromatographic process for three HETP values ($H_1 < H_2 < H_3$).

(2) If component B decreases α_A, the time of overlapping is shortened.

(3) If component A increases α_B, the time of overlapping is shortened.

(4) If component A decreases α_B, the time of overlapping is prolonged.

In cases 1 and 4, we have the phenomenon of "spot approaching" and in cases 2 and 3, the phenomenon of "spot repulsion".

In Fig. 2, the area $A \cap B$ represents the amount of component B that influences component A, and *vice versa*. With a decrease in the ratio $(A \cap B)/A$ or $(A \cap B)/B$, this influence becomes smaller.

The sharpness of the spot is directly proportional to the number of theoretical plates, or inversely proportional to the HETP value for each component. When the number of theoretical plates increases, for the same distance between the peaks for components A and B, the ratio $(A \cap B)/A$ diminishes, because the peaks are narrower.

Fig. 3 shows the change in α_A during the chromatographic process, when the ratio of the amounts of components A and B is constant, for three HETP values: $HETP_1 < HETP_2 < HETP_3$. The curvature of the lines decreases with the increase in HETP value, and the differential form can be given by the expression:

$$\left(\frac{d^2\alpha}{dx^2}\right)_{H_1} > \left(\frac{d^2\alpha}{dx^2}\right)_{H_2} > \left(\frac{d^2\alpha}{dx^2}\right)_{H_3} \tag{4}$$

The real R_F value is a function of $\bar{\alpha}$, which is obtained from the following equation:

$$\bar{\alpha} = \frac{\int_s^{x_A} f(\alpha)dx}{x_A} \tag{5}$$

Substitution of the expression for $\bar{\alpha}$ in the equation of MARTIN AND SYNGE gives:

$$\Delta R_F = \frac{A_M}{A_S}\left[\alpha_A - \frac{\int_s^{x_A} f(\alpha_A)dx}{x_A}\right] R_F \cdot R_F' \tag{6}$$

In mathematical calculations it is better to convert the R_F values into R_M values, because the relation for the latter values is linear:

$$\Delta R_M = \log|\alpha| - \log|\bar{\alpha}| \tag{7}$$

or

$$\alpha = \bar{\alpha} \cdot e^{-\Delta R_M \cdot 2{,}3} \tag{8}$$

From the diagram in Fig. 3 it can be concluded that the difference between $\bar{\alpha}$ and α diminishes when the plate number increases. By extrapolation it is possible to obtain α:

$$\alpha = \lim_{\Delta R_M \to 0} \bar{\alpha} \tag{9}$$

As is shown in Fig. 4, and in accordance with eqn. (8), the approximate relation between $\Delta R_{M(A)}$ and the amount of component B, M_B, can be given by eqn. (10), in which K is a constant:

$$\Delta R_{M(A)} = Km_B \tag{10}$$

or

$$R_{M(0)(A)} - R_{M(A)} = Km_B$$

which is valid for all four cases.

From eqn. (10) it is evident that if $R_{M(i)(A)}$ values for various values of $m_{i(B)}$ are empirically determined, the $R_{M(0)}$ value can be obtained by means of statistical methods (either graphical or mathematical):

$$R_{M(0)} = \frac{(\Sigma R_{M(i)}) \cdot (\Sigma m_i^2) + (\Sigma R_{M(i)} \cdot m_i) \cdot (\Sigma m_i)}{N(\Sigma m_i^2) - (\Sigma m_i)^2} \tag{11}$$

where N is the number of measurements,

$R_{M(0)}$ is converted into the standard $R_{F(0)}$ value, by means of a conversion table or the equation $R_M = \log[1/R_F - 1]$.

TABLE I

FIRST PLATE

Amounts in μg.

	I	*II*	*III*	*IV*	*V*	*VI*
Component A	10	10	10	10	10	10
Component B	2	5	8	10	15	20

TABLE II

SECOND PLATE

Amounts in μg.

	I	*II*	*III*	*IV*	*V*	*VI*
Component A	2	5	8	10	15	20
Component B	10	10	10	10	10	10

EXPERIMENTAL

The experiments were performed according to the usual method[2]. On the plates (20 × 20 cm) the adsorbent (Silica Gel H; thickness, 0.25 mm) was activated at 120° for 20 min. The chromatograms were developed with toluene. The substances investigated were: component B—Sudan Red III (R_F 0.220); component A—Fettrot (R_F 0.403). The mixture was applied in the ratios given in Tables I and II.

The R_M values obtained are given in Fig. 4, where it can be seen that the change

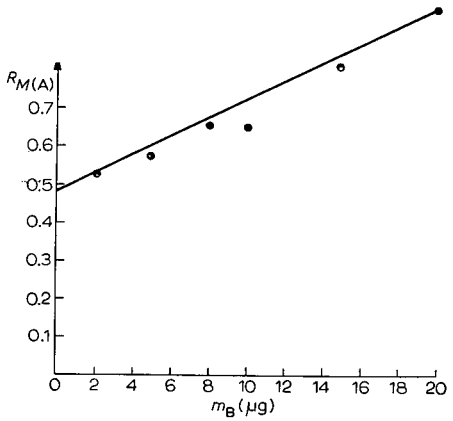

Fig. 4. Change in $R_{M(A)}$ *versus* amount of component B.

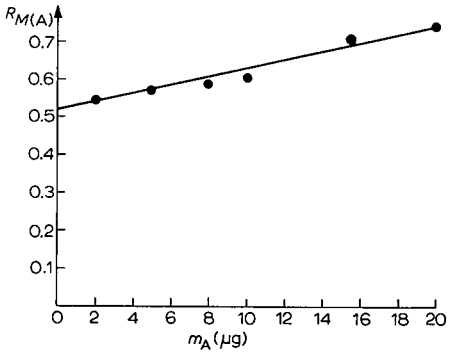

Fig. 5. Change in $R_{M(A)}$ *versus* amount of component A.

in $R_{M(A)}$ is proportional to the amount of component B, thus establishing eqn. (10).

Fig. 5 shows that when the amount of component B is kept constant, the change in the R_M value is proportional to the amount of component A, because the ratio $(A \cap B)/A$ increases with the increase in the amount of component A.

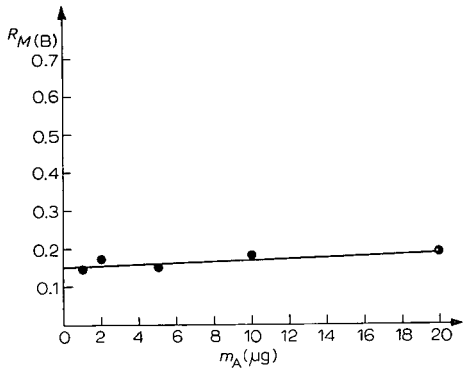

Fig. 6. Diagram showing that the presence of component A does not influence $R_{M(B)}$.

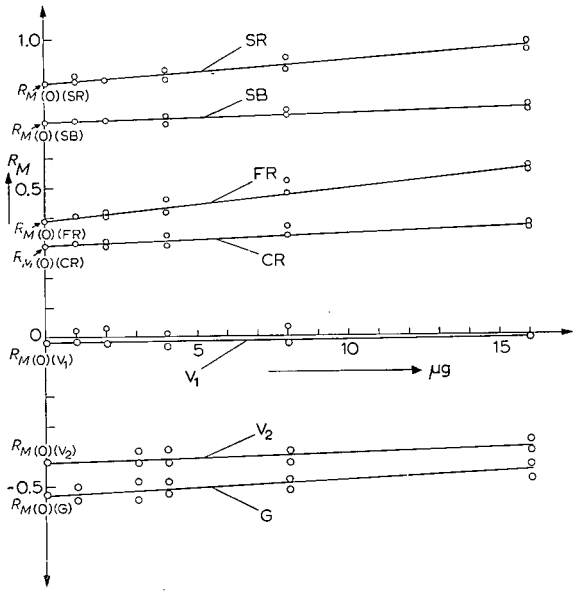

Fig. 7. Extrapolation method for calculation of the $R_{M(0)}$ values of seven components of a mixture. For abbreviations, see Table III.

The presence of component A does not affect the distribution coefficient, α_B, as is shown in Fig. 6. The effect of component A on component B was described previously as case (3).

The R_M values obtained are given in Fig. 7. The corresponding $R_{M(0)}$ values have been converted into $R_{F(0)}$ values, which are given in Table III.

TABLE III

THIRD PLATE

Amounts in μg.

Substances	*I*	*II*	*III*	*IV*	*V*	$hR_{F(0)}$
Sudan Red III (SR)	16	8	4	2	1	12.4
Sudan Blue (SB)	16	8	4	2	1	16.0
Fettrot (FR)	16	8	4	2	1	29.0
Ceres Red (CR)	16	8	4	2	1	33.3
Sudan Violet (V_1)	16	8	4	2	1	51.2
Sudan Violet (V_2)	16	8	4	2	1	72.6
Fettreingelb (G)	16	8	4	2	1	77.2

DISCUSSION AND CONCLUSION

The working conditions should be constant throughout, *e.g.* the areas of the applied spots, the saturation of the chamber and the uniformity of the layer. It is important to keep the area of the applied spot constant, which is possible only by always applying the same volume of solution. The saturation of the chamber should also be complete in order to avoid edge effects.

The above arguments are only valid when the zone width is so small that the distance substance A has to go to traverse substance B is negligible. Obviously, if the zone is wider (*e.g.* in preparative work), not only the R_F value but also the shape of the spot will change because the tail of zone A will remain in contact with zone B much longer than the front.

This method can be adopted not only to obtain the "real" R_F value, but also to standardize the R_F value of compounds which change on increasing the amount of compound. In this case the standard R_F value can be given as $R_{F(0)}$ (when the amount of the compound tends toward zero).

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CHROM. 3661

UNTERSUCHUNGEN ZUR ELEKTROSTATISCHEN AUFLADUNG TRITIUMMARKIERTER DÜNNSCHICHTCHROMATOGRAMME WÄHREND IHRER AUSMESSUNG AM FENSTERLOSEN PROPORTIONALZÄHLROHR

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SUMMARY

Investigation of the electrostatic charge of tritium-labelled thin-layer chromatograms during their measurement with a windowless proportional counter

The positive surface potentials, which occur when measuring the activity of insulated ^{3}H-labelled thin-layer chromatograms with windowless counters, are characterized as regards magnitude and development as well as by their influence on the size of the error of the activity measurements. A measuring arrangement is described, whereby the occurrence of this type of electrostatic effect is prevented by continuous grounding of the charge. In this way the count rate constancy and reproducibility necessary for a quantitative evaluation of the labelled thin-layer chromatograms is guaranteed without loss of efficiency.

Die Vorlage halb- oder nichtleitender Aktivitätsträger kann bei fensterloser Messung von Tritium mit Proportionalzählrohren zu Feldstörungen bzw. zeit- und aktivitätsabhängigen elektrostatischen Aufladungserscheinungen an der Trägeroberfläche führen. In Form sich daraus ableitender Zählratenänderungen üben diese einen mehr oder weniger grossen Einfluss auf Effektivität und Reproduzierbarkeit der Messergebnisse aus. Für Messungen von ^{3}H-Festpräparaten eliminierten deshalb BANKS und Mitarbeiter[1] die elektrostatische Aufladung durch Graphitzugaben zum Messpräparat und erreichten eine 10-fach geringere Standardabweichung. SPANG UND GEBAUHR[2] erhöhen die Leitfähigkeit ihrer auf Papier isolierten Messproben durch Aufdampfen einer Silberschicht.

SCHÜRFELD UND WEGNER[3] und später auch SCHMIDT[4] prüften die Verhältnisse für ausserhalb des Detektors angeordnete ^{3}H-Aktivitätsträger mit geringem Leitvermögen, wie dies bei der fensterlosen Ausmessung von Radiodünnschicht- und Radiopapierchromatogrammen mit Gasdurchflusszählrohren (Schlitzblende) gegeben ist. Es konnte gezeigt werden[3], dass neben den im empfindlichen Zählervolumen direkt erfassten β-Teilchen in sehr beträchtlichem Masse extern gebildete negative, sekundäre Ladungsträger in die Registrierung eingehen. Bei Verwendung nichtleitender Aktivitätsträger werden letztere jedoch durch die in Abhängigkeit von der Gasver-

stärkung sich ausbildende positive Oberflächenladung des Trägers partiell abgesaugt und von der Registrierung ausgeschlossen. Den hieraus resultierenden Zählratenänderungen kann durch Überführung des Trägers in einen leitfähigen Zustand oder durch Abfangen der extern gebildeten negativen sekundären Ladungsträger begegnet werden. Während SCHMIDT[4,5] für die Messung von ^{3}H-markierten Papierchromatogrammen mit offenem Proportional-Doppelzählrohr die Leitfähigkeit des Trägermaterials durch Aufsprühen einer Graphit-Suspension erhöht und so bei einem Absorptionsverlust von nur 7 % eine Papieraufladung verhindert, schliessen SCHÜRFELD UND WEGNER[3] bei der Untersuchung nichtleitender Modell-Präparate (^{3}H-Auftragung auf Glasplatte) die extern gebildeten negativen sekundären Ladungsträger durch den Aufbau eines künstlichen elektrischen Feldes (Gitterblende) vor der Schlitzblende von der Registrierung aus. In dieser Anordnung steht jedoch dem Vorteil der Zählratenkonstanz ein durch die totale Eliminierung der sekundären und wahrscheinlich partiellen Absorption der primären Ladungsträger hervorgerufener starker Empfindlichkeitsabfall um den Faktor 5–10 gegenüber. Zusätzlich erhöht sich die Abstandsempfindlichkeit (Abstand Blende–Chromatogrammoberfläche) der Messanordnung beträchtlich.

Untersuchungen zur Fehlerbreite quantitativer radiodünnschichtchromatographischer Messungen[6] veranlassten uns, die an regulären tritiummarkierten Dünnschichtchromatogrammen vorliegenden Verhältnisse mit dem Ziel einer vollständigen Eliminierung der elektrostatischen Aufladung bei unveränderter Messeffektivität und Abstandsempfindlichkeit zu prüfen. Wie vergleichende Impuls- und Potentialmessungen unter Verwendung isolierender und leitender Chromatogrammträger ergaben, kann durch intensiven und grossflächigen Kontakt der Dünnschichtchromatogramme mit erdableitenden Unterlagen die Bildung statischer Aufladungen auf Chromatogrammen ausgeschlossen und auch bei Tritium ohne jeden Empfindlichkeitsabfall die für eine quantitative Auswertung erforderliche Konstanz der Zählraten erreicht werden.

MATERIAL UND METHODEN

Die in üblicher Weise auf Glasplatten (10 × 20 cm) aufgebrachten Kieselgel-Schichten (Kieselgel G Merck) besassen eine ausgemessene Schichtdicke von 200 μm $\pm$ 5 %[7]. Als tritiumhaltiges Material fand Digitoxin–(u–^{3}H)[8] mit einer spezifischen Aktivität von 288 mC/mMol. Verwendung. Um eine gleichmässige und reproduzierbare Verteilung des Nuklides in der Trägerschicht und damit an der Oberfläche zu gewährleisten, wurden sämtliche Untersuchungen nur an chromatographierten DC-Platten vorgenommen (Äthylacetat–Methanol-Systeme).

Die Aktivitätsmessung der Chromatogramme erfolgte an einem mit dem Gasdurchflusszähler VA-Z-530* (mit modifizierter Zähldrahtanordnung) ausgestatteten Radiodünnschichtchromatographen[6]. Der Abstand zwischen Schlitzblende (2 mm) und Chromatogrammoberfläche betrug gleichbleibend 0.5 mm[7]. Messdaten: Eingangsempfindkichkeit 2 mV, Arbeitsspannung (U_H) 3.15 kV, Methanfluss 2 l/h; Scanner- und Bandschreiber-Geschwindigkeit: 600 mm/h.

* VEB Vakutronik, Dresden.

Zur direkten Ausmessung der elektrostatischen Oberflächenladung der Chromatogramme fand entsprechend der in Fig. 1 dargestellten Schaltung ein Schwingkondensatorelektrometer vom Typ VA-J-51* Verwendung. Der Potentialabgriff auf

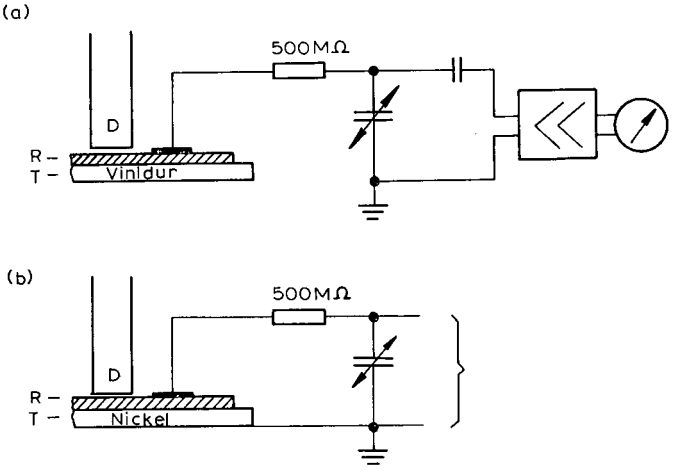

Fig. 1. Schaltskizze zur Ausmessung der elektrostatischen Aufladung an Chromatogrammoberflächen. Radiodünnschichtchromatograph: D = Detektor VA-Z-530; R = Radiodünnschichtchromatogramm; T = Chromatogramm-Transporteinrichtung. a) Chromatogramm-Träger (= Transportschlitten des Radiodünnschichtchromatographen) isoliert; Vinidurplatte. b) Chromatogramm-Träger mit Erdableitung; Nickelplatte.

der Schichtoberfläche erfolgte mit einer 2 × 4 cm grossen Kupferfolie. Der Eingangswiderstand der Messanordnung betrug $10^{11}\,\Omega$. Als nichtleitender Chromatogramm-Träger (Bestandteil der Transporteinrichtung des Radiodünnschichtchromatographen) diente Vinidur (Fig. 1a), während durch intensiven breitflächigen Kontakt (Wasserfilm) des Chromatogramms mit einer separat geerdeten Nickelplatte (14 × 24 cm) die Möglichkeit der Potentialableitung (Fig. 1b) geprüft wurde.

ERGEBNISSE

Die Untersuchungen gingen von den in Fig. 2 und 3 wiedergegebenen Befunden aus. Bei fehlender Ableitung, d.h. bei Chromatogramm-Unterlagen aus isolierendem Material (Vinidur), resultiert bei fortlaufender, wiederholter Ausmessung ein und desselben Tritiumpeaks (Fig. 2a) ein eindeutiger zeitabhängiger Abfall der Flächen- bzw. maximalen Impulshöhenwerte (I_{max}). Während der Gesamtmessdauer für eine 5-fache Ausmessung von 6 min sinken die I_{max}-Werte von 3600 auf 2700 Imp. × min^{-1} (= 75 % des Ausgangswertes) ab, wobei die Differenz zwischen der 1. und 2. Integration mit 13 % am höchsten liegt. Ein analoges Bild ergibt sich für die entsprechende Darstellung einer stationären ^{3}H-Quelle gegen die Zeit (Fig. 3a). Dagegen beweist die vergleichende Messung unter Verwendung einer Nickelplatte mit Erdableitung als Chromatogramm-Unterlage, sowohl für die wiederholte Aufzeichnung ein und desselben Peaks (Fig. 2b; Gesamtmesszeit für 4 Messungen: 6 min) als auch bei Vorlage

* VEB Vakutronik, Dresden.

einer stationären ^3H-Quelle (Fig. 3b), dass durch eine in dieser Anordnung vorgenommene Ableitung die Ausbildung einer statischen Aufladung der Chromatogramm-Oberfläche vermieden und damit die erforderliche Zählratenkonstanz erzielt werden

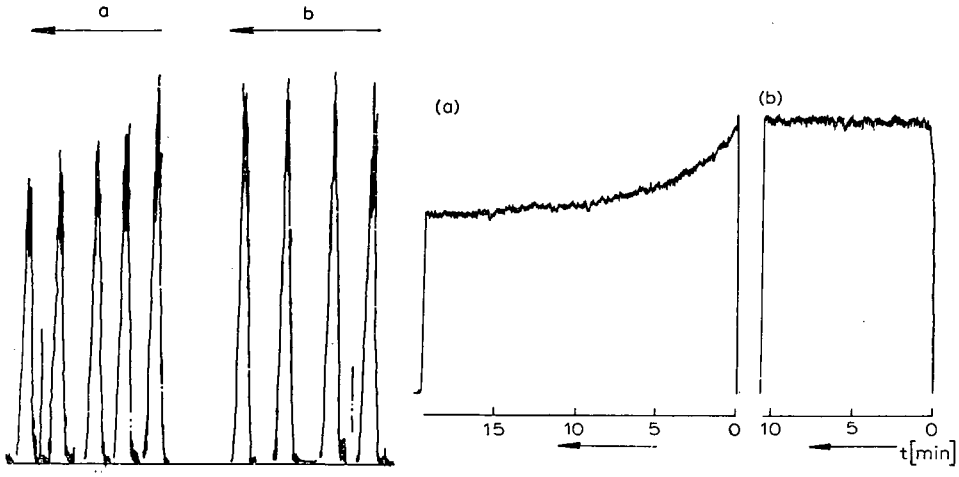

Fig. 2. Aktivitätsverteilungskurven eines ^3H-markierten Dünnschichtchromatogramms; aufgenommen (a) ohne und (b) mit Ableitung (wiederholte Aufzeichnung eines ^3H-Peaks; Integrationsbereich: 6×10^3 Imp. $\times$ min^{-1}).

Fig. 3. Vergleichende Entwicklung der I_{max}-Werte einer stationären ^3H-Quelle gegen die Zeit (Integrationsbereich: 6×10^3 Imp. $\times$ min^{-1}); (a) ohne; (b) mit Ableitung.

kann. Hiervon ausgehend, wurden die Grösse und Charakteristik der Oberflächenladung (U_{Ch}) in Funktion der Zeit (t), Aktivitätsmenge (A) und Zählrohrspannung (U_H) sowie vergleichend die Entwicklung der maximalen Impulsdichte in verschiedenen Aktivitätsniveaus mit und ohne Ableitung untersucht.

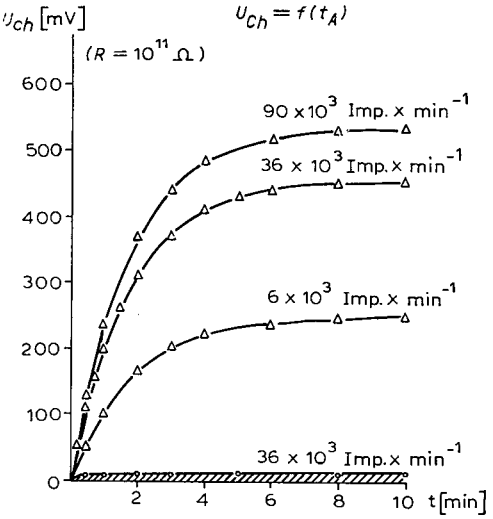

Fig. 4. Elektrostatische Aufladung der Chromatogrammoberfläche (U_{Ch}) in Abhängigkeit von Messzeit (t) und Aktivität (A). (△—△) ohne Ableitung; (○—○) mit Ableitung.

$U_{Ch} = f(t_A)$

Die Potentialmessung erfolgte nach sorgfältiger Abschirmung bei einem Eingangswiderstand des Messgerätes von $R = 10^{11}\,\Omega$. In Übereinstimmung mit der Impulsratenentwicklung ist bei fehlender Ableitung nach Zuschaltung der Zählrohrspannung ein sofortiger steiler Anstieg der U_{Ch}-Werte zu verzeichnen, der etwa bis zur 4-Minute anhält (Fig. 4). Der nach 10 min erreichte Sättigungswert beträgt bei 36×10^3 Imp. $\times$ min^{-1} 450 ± 18 mV ($\bar{x} \pm s$; $n = 3$)*.

Der aus den 1, 2, 4 und 10 Minuten-Werten von 3 separaten Messreihen (36×10^3 Imp. $\times$ min^{-1}) gebildete Mittelwert der relativen Standardabweichungen der Einzelwerte von $\pm$ 4.3 % unterstreicht den bei gleichbleibenden Bedingungen konstanten Verlauf der Potentialbildung. Der Wert des Anstiegswinkels sowie die absolute Höhe der Potential-Sättigung (in mV) steht, wie vergleichende Messungen mit zwei weiteren Aktivitätsniveaus von 6 und 90×10^3 Imp. $\times$ min^{-1} zeigen, in Abhängigkeit von der ^{3}H-Aktivität der Vorlage. Im Gegensatz hierzu unterbleibt bei Verwendung eines leitenden Trägers der Aufbau einer elektrostatischen Chromatogramm-Aufladung. Das Oberflächenpotential erhöht sich unter einer Aktivität von 36×10^3 Imp. $\times$ min^{-1} nur geringfügig auf maximal 12 mV.

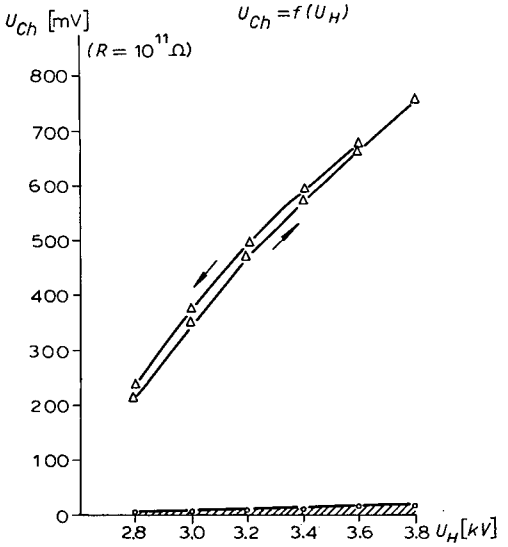

Fig. 5. Elektrostatische Chromatogramm-Aufladung (U_{Ch}) in Abhängigkeit von der Zählrohrspannung (U_H); gemessene Aktivität: 36×10^3 Imp. $\times$ min^{-1}; Einstellungsbewegung zwischen den Messpunkten: $\approx$ 10 mV $\times$ min^{-1}; (△—△) ohne Ableitung; (○—○) mit Ableitung.

$U_{Ch} = f(U_H)$

Messungen zum Einfluss des Gasverstärkungsfaktors (Fig. 5) auf die Ausbildung des Oberflächenpotentials zeigen für 36×10^3 Imp. $\times$ min^{-1} im Bereich von 2.8–3.8 kV (U_H) bei fehlender Ableitung mit einem Anstieg von 200 auf 750 mV (U_{Ch}) eine fast lineare Beziehung der U_{Ch}-Werte, die auch bei absteigender Messung

* $s = \pm \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}}$.

nur eine unbedeutende Verschiebung erfahren. Die Einstellungsbewegung zwischen den Messpunkten beträgt ≈ 10 mV $\times$ min^{-1}. Die entsprechenden Ableitungswerte bewegen sich von 5 auf 17 mV.

$I_{max} = f(t_A)$

Zur Frage des Einflusses der dargestellten Potentialbildung auf die Zählratenkonstanz und damit auf die Fehlerbreite radiodünnschichtchromatographischer Messungen wurde die Entwicklung der Impulsdichten in 3 verschiedenen Aktivitätsniveaus mit und ohne Ableitung gegen die Zeit verfolgt (Fig. 6). Während eine Potentialableitung die Konstanz der Zählraten innerhalb der zulässigen Fehlerbreite

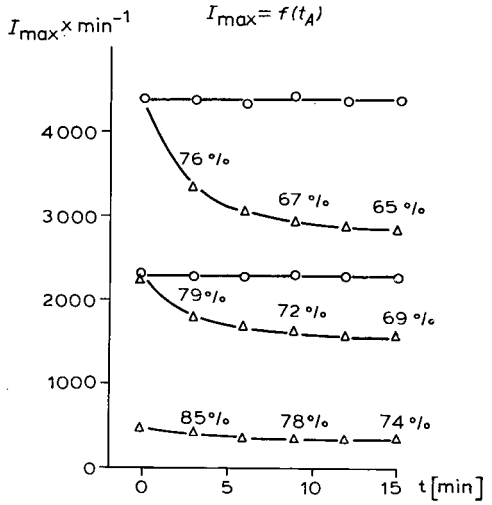

Fig. 6. Vergleichende Entwicklung der I_{max}-Werte 3 verschiedener ^{3}H-Aktivitätsniveaus gegen die Zeit mit (O—O) und ohne (△—△) Ableitung; prozentuale Angaben bezogen auf Ausgangswert.

gewährleistet, bewirkt eine Chromatogramm-Aufladung in allen untersuchten Aktivitätsbereichen bereits nach einer Messdauer von 2 min eine negative Abweichung vom Ausgangswert von >13 %. In Übereinstimmung mit den Potentialmessungen wird die Geschwindigkeit sowie die absolute Grösse des Abfalls durch das vorgegebene Aktivitätsniveau beeinflusst. Nach 15 min beträgt die Abweichung je nach Aktivität minus 25–35 %.

DISKUSSION

Die Isolierung tritiummarkierter Dünnschichtchromatogramme durch Verwendung nicht oder schlecht leitender Trägereinrichtungen (Vinidur) (Fig. 1a) führt im Verlauf ihrer Ausmessung mit fensterlosen Gasdurchflusszählrohren in Abhängigkeit von Messzeit, Aktivitätsmenge (Fig. 4) und Gasverstärkungsfaktor (Fig. 5) zur Ausbildung unterschiedlich grosser, positiv geladener Oberflächenpotentiale. Die hierbei durch Bindung der sekundären negativen Ladungsträger hervorgerufenen Zählratenverluste können je nach vorliegendem Aktivitätsniveau bis 35 % des Ausgangs-

wertes betragen (Fig. 6). Bei konstanter Gasverstärkung besteht zwischen der Charakteristik direkter Potentialmessungen an der Chromatogrammoberfläche und der Entwicklung der Impulsdichte eine generelle, zeitabhängige Übereinstimmung. Der Potentialanstieg, wie auch der ihm entsprechende Zählratenabfall, erreicht nach rascher Anfangsentwicklung nach 10 bzw. 15 min einen Grenzwert, der, ebenso wie der Anstiegswinkel, in seiner Grösse wesentlich vom vorliegenden Aktivitätsniveau beeinflusst wird. Der demonstrierte Zählratenabfall, der nach 3 min bereits bei 15–25 % liegt, geht damit in dieser Grössenordnung in die Fehlerbreite der Scanner-Messungen ^{3}H-markierter Dünnschichtchromatogramme ein (Fig. 2a) und kann bei Messungen mit kleinen Vorschubwerten (60–200 mm/h) sogar innerhalb der Impulsmessung ein und desselben Peaks (bei durchschnittlichen Fleckendurchmesser von 5 mm Messdauer: 5–1.5 min) zur Auswirkung gelangen. Damit wird die Reproduzierbarkeit quantitativer Aussagen weitgehend in Frage gestellt.

Für die somit erforderliche Eliminierung der elektrostatischen Aufladung kam eine Chromatogramm-Behandlung mit Graphit[4] aus Gründen einer möglichen Beeinträchtigung der an die Aktivitätsmessung sich im Bedarfsfall anschliessenden quantitativ-chemischen Bestimmungen bzw. präparativen Isolierung der markierten Verbindungen nicht in Betracht. Ebenso erschien uns die Verwendung einer Gitterblende[3] wegen des damit verbundenen 5- bis 10-fachen Empfindlichkeitsverlustes (durch Ausschaltung der sekundären Ladungsträger) sowie der wesentlich erhöhten Abstandsempfindlichkeit nicht vertretbar.

Unter der Voraussetzung einer partiellen Leitfähigkeit des Kieselgel G infolge seines Gehaltes an Metall-Ionen[9], besonders Eisen, und der Annahme einer gewissen Beweglichkeit der Oberflächenladung über kurze Glasabschnitte (Feuchtigkeitsfilm etc.), prüften wir die Möglichkeit der direkten und kontinuierlichen Erdableitung des Oberflächenpotentials durch optimalen Flächenkontakt mit einem an der Unterseite des Chromatogramms befindlichen Erdleiter. Durch Impulsdichtemessungen mit bewegter (Fig. 2b) und stationärer ^{3}H-Quelle (Fig. 6) sowie durch direkte Potentialmessungen (Fig. 4) konnte gezeigt werden, dass in dieser Anordnung der Aufbau einer positiven Oberflächenladung bei ^{3}H-markierten Dünnschichtchromatogrammen unterbunden werden kann, und die für ihre quantitative Auswertung erforderliche Zählratenkonstanz gewährleistet ist. Da unter diesen Bedingungen die sekundären Ladungsträger der Registrierung nicht entzogen werden, bleibt die Messeffektivität im Gegensatz zur Anordnung mit vorgeschalteter Gitterblende[3] unverändert erhalten. Die Abstandsempfindlichkeit erhöht sich nicht.

DANK

Fräulein S. JÜNGLING danke ich für ihre wertvolle Mitarbeit bei der Durchführung dieser Untersuchungen. Herrn Chem. Ing. O. RISTAU bin ich für seine Hinweise sehr zu Dank verpflichtet.

ZUSAMMENFASSUNG

Die bei fensterloser Aktivitätsmessung isolierter ^{3}H-markierter Dünnschichtchromatogramme auftretenden positiven Oberflächenpotentiale werden in Grösse und Entwicklung sowie hinsichtlich ihres Einflusses auf die Fehlerbreite der Aktivitäts-

messung charakterisiert. Es wird über eine Messanordnung berichtet, die durch kontinuierliche Ableitung der Ladung das Auftreten derartiger elektrostatischer Effekte verhindert und dadurch bei gleichbleibender Messeffektivität die für eine quantitative Auswertung radiodünnschichtchromatographischer Messungen erforderliche Zählratenkonstanz und Reproduzierbarkeit gewährleistet.

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CHROM. 3654

QUANTITATIVE MICRODETERMINATION BY THIN LAYER CHROMATOGRAPHY OF THE PHOTOPRODUCTS INVOLVED IN THE PHOTOCYCLIZATION OF *cis*-3-STYRYLPYRIDINE

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SUMMARY

The quantitative determination of the products involved in the photocyclization of *cis*-3-styrylpyridine by thin layer chromatography is described. The method may find wide application in following the photoreaction pathway at any time by the quantitative determination of the two photocyclization products and of the two *cis* and *trans* isomers. The determination is obtained by spectrophotometric microanalysis. Using a concentration of $5.5 \cdot 10^{-4}$ and a 254 mμ exciting line, the ratio of the two benzoquinolines is constant at about 5.3 in favour of the 5,6-benzoisoquinoline. It increases with irradiation at 313 mμ.

Thin layer chromatography (TLC) in conjunction with quantitative spectrometric microanalysis was investigated for the determination of the photocyclization products of *cis*-3-styrylpyridine. For the latter purpose an analytical method was necessary which allows the separate determination of the various products involved in the reaction. In a previous paper[1], it was shown that in the photocyclization of *cis*-3-styrylpyridine, in addition to 5,6-benzoisoquinoline(II), as found by LOADER[2], small amounts of 7,8-benzoquinoline(I) are also formed (Fig. 1).

The purpose of the present investigation was to establish the ratio of 5,6-benzoisoquinoline to 7,8-benzoquinoline as a function of time. The influence of the wavelength of the exciting light was also studied. The analysis of the irradiated solution was complicated by the presence of the two geometric isomers since *cis–trans* photoisomerization takes place in parallel with the photocyclization[3]. The photocyclization of the stilbenes[4], styrylpyridines[1,2], styrylthiophenes[5] and azobenzenes[6] is known and has been intensively studied during the last few years. However only a few authors were interested in the formation of cyclized isomeric photoproducts as a consequence either of the presence in the ring of a heteroatom as described in Fig. 1 or of the presence of a substituent as described in Fig. 2. Of these authors, MALLORY[4], LEWIS[6] and CARRUTHERS[5] on investigating the latter type of isomerism found that the different isomeric products were formed in nearly equivalent quantities.

PERKAMPUS[7], LOADER[8], and the present authors[1,9], examined the former type of isomerism (different position of the heteroatom), and found that the different

isomeric products were formed in highly variable amounts, in agreement with our preliminary results[1]. Some authors explained the larger yield of one product with respect to another on the basis of theoretical calculations of charge density and of localization energy in the different reactive positions both in the ground state[8,10] and in the first excited state[9,11]. In order to compare the experimental data with the theoretical calculations an accurate analytical procedure was developed. In particular we employed TLC using Eastman Chromagram sheets instead of the usual silica

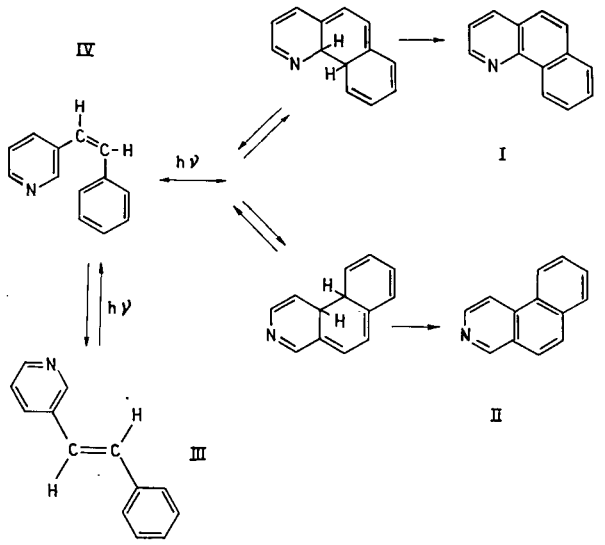

Fig. 1. Scheme of photocyclization and isomerization of 3-styrylpyridine.

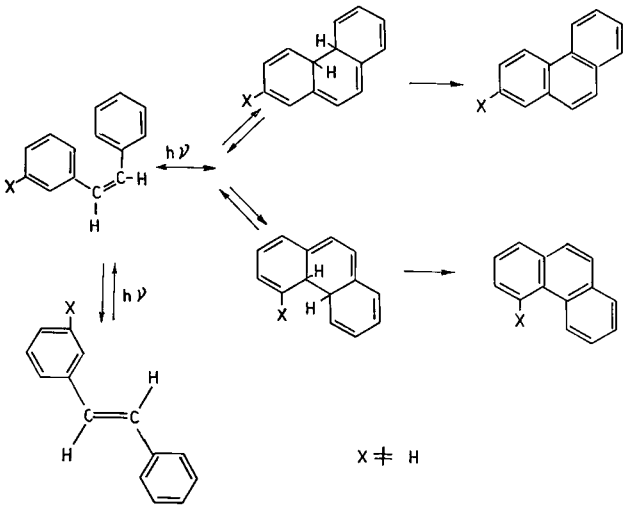

Fig. 2. Scheme of photocyclization of 3-substituted stilbene.

glass plates. We preferred to use these Chromagram sheets because of their high reproducibility and sensitivity, and because of the facility with which the separated components can be recovered. This fact is very important for a quantitative analysis.

EXPERIMENTAL

Preparation of standard compounds

The 3-styrylpyridines, *cis* and *trans*, the 7,8-benzoquinoline and the 5,6-benzoisoquinoline were prepared as described by GALIAZZO[1,12]. In all cases the purity of the products was also determined by I.R. and U.V. spectra, and by C, H and N analyses.

Materials

Eastman Chromagram sheets (*K 301 V*). These are precoated sheets of polyethylene terephthalate coated with a layer of about 100 μ silica gel with polyvinyl alcohol as a binder.

Chromatography tanks. Desaga GmbH, Heidelberg.

Micropipettes. A. E. Pedersen, Denmark.

Solvents. Ethyl ether, methanol, *n*-hexane (Erba) specially prepared for chromatography and tested for absorption in the ultraviolet. Dimethylformamide was dried over phosphorus pentoxide and was then fractionally distilled in the dark and under reduced pressure immediately before use.

Spectrophotometer. Beckman DU.

Quartz cell. U.V. sources, 4 cm path-length. Mineralight, low pressure mercury lamp equipped with a 254 mμ filter; Osram, high pressure (HBO-200) mercury lamp with 313 mμ filter.

Procedure

The *cis*-3-styrylpyridine in *n*-hexane (about 10^{-4} mole/l) was irradiated in a quartz cell with a U.V. lamp for times varying from ½ h to 15 h. A fixed support kept the cell at a constant distance.

The spotted amounts of irradiated solutions were chosen such that they have an optical density between 0.600 and 0.200 for every separated compound. When the volumes of solution were too large, the solvent was evaporated under reduced pressure to a final volume of a few microliters (about 10 μl).

After irradiation, duplicate samples of the solution, containing the three photoproducts and the reagent, were spotted in an appropriate quantity by means of a micropipette. At the same time known amounts of the test compounds corresponding to each component of the reaction mixture were spotted. The sheets were placed in the chromatography tank, previously saturated with the solvent vapours, ethyl ether–dimethylformamide (99:1) for 1 h[13–15]. The chromatograms were developed by the usual ascending technique. The sheets were removed when the solvent front reached 10 cm: running time 60 min. The spots were located under 366 mμ U.V. light, cut out from the sheets in strips of identical size and extracted with 5 ml of methanol. After 15 min the solution was centrifuged at 15,000 r.p.m. for 10 min to remove the last traces of silica gel, the optical density was measured at the absorption maximum of each compound, employing cells of 1 cm path-length. The complete spectrum of every compound was measured simultaneously. The amounts of the substances were

calculated by reference to data obtained with known amounts of control, after subtracting the reading given by an equal area of unstained sheet at locations on the plate with precisely equivalent R_F values.

RESULTS AND DISCUSSION

The R_F values are given in Table I. Since these compounds have high extinction coefficients, a suitable spectrophotometric reading can also be made with quite low concentrations. Table I shows that, within the limits of accuracy of the measurements, Beer's law is obeyed up to a concentration at least as high as $6.1 \cdot 10^{-5} M$. Undoubtedly silica gel is the most suitable absorbent in the quantitative analysis of the

TABLE I

RECOVERY OF THE REACTION PRODUCTS FROM SILICA GEL LAYERS AND R_F VALUES[a]

	ε max 10^4 [g]	*Absorbance*			*Percent recovery*	$R_F \times 100$[h]
		Sample	*Blank*	*Net*		
7,8-Benzoquinoline[b]	2.38					82
Solution determined directly[c]	(max = 265 mμ)	0.537		0.537	100	
Spotted on plate and developed		0.515	0.011	0.504	94	
		0.511	0.008	0.503	94	
		0.513	0.015	0.498	93	
5,6-Benzoisoquinoline[b]	5.38					42
Solution determined directly[d]	(max = 249.5 mμ)	0.404		0.404	100	
Spotted on plate and developed		0.381	0.000	0.381	94	
		0.380	0.005	0.375	93	
		0.375	0.003	0.372	92	
trans-3-Styrylpyridine[b]	2.34					54
Solution determined directly[e]	(max = 292 mμ)	0.523		0.523	100	
Spotted on plate and developed		0.487	0.003	0.484	93	
		0.494	0.006	0.488	94	
		0.487	0.007	0.480	92	
cis-3-Styrylpyridine[b]	1.05					67
Solution determined directly[f]	(max = 277 mμ)	0.352		0.352	100	
Spotted on plate and developed		0.332	0.007	0.325	92	
		0.340	0.009	0.331	94	
		0.339	0.010	0.329	93	

[a] Eluent system: ethyl ether–dimethylformamide.
[b] Stock solution: 7,8-benzoquinoline, 10.094 mg; 5,6-benzoisoquinoline, 13.457 mg; *trans*-3-styrylpyridine, 10.058 mg; *cis*-3-styrylpyridine, 10.150 mg; respectively, were dissolved in methanol in a 10 ml volumetric flask.
[c] Aliquot of 20 μl was diluted to 5 ml.
[d] Aliquot of 10 μl was diluted to 5 ml.
[e] Aliquot of 20 μl was diluted to 5 ml.
[f] Aliquot of 30 μl was diluted to 5 ml.
[g] Average of six determinations.
[h] Average of ten determinations.

TABLE II

LIGHT-INDUCED (254 mμ) CYCLIZATION AND ISOMERIZATION OF *cis*-3-STYRYLPYRIDINE. PERCENTAGE AND RATIO OF THE REACTION PRODUCTS AS A FUNCTION OF THE TIME

Conc. ≈ $5 \cdot 10^{-4}$ *M*. Compound I = 7,8-benzoquinoline; compound II = 5,6-benzoisoquinoline; compound III = *trans*-3-styrylpyridine; compound IV = *cis*-3-styrylpyridine.

Time (min)	*Percentage of the reaction products (weight %)*				*Compound II / Compound I*	*Total percent recovery*
	Compound I	*Compound II*	*Compound III*	*Compound IV*		
30	1.43	7.63	18.07	69.27	5.3	96.40
120	3.85	21.09	20.90	45.65	5.4	91.49
210	5.85	32.14	15.30	37.00	5.5	90.29
300	7.40	39.90	11.61	31.99	5.4	90.90
420	9.38	50.10	7.30	24.24	5.3	91.02
525	10.70	58.77	4.54	17.60	5.5	91.61
735	12.76	67.10	1.75	8.26	5.3	89.87
900	13.45	69.92	0.50	5.63	5.2	89.50

TABLE III

LIGHT-INDUCED (313 mμ) CYCLIZATION AND ISOMERIZATION OF *cis*-3-STYRYLPYRIDINE: PERCENTAGE AND RATIO OF THE REACTION PRODUCTS AS A FUNCTION OF THE TIME

Conc. ≈ $5 \cdot 10^{-4}$ *M*. Compound I = 7,8-benzoquinoline; compound II = 5,6-benzoisoquinoline; compound III = *trans*-3-styrylpyridine; compound IV = *cis*-3-styrylpyridine.

Time (min)	*Percentage of the reaction products (weight %)*				*Compound II / Compound I*	*Total percent recovery*
	Compound I	*Compound II*	*Compound III*	*Compound IV*		
210	3.35	25.13	4.59	58.17	7.5	91.24
300	4.47	34.17	4.17	48.86	7.6	91.67
330	4.80	36.69	3.51	46.68	7.6	91.68
525	7.83	59.75	1.70	21.24	7.6	90.52
735	9.48	72.63	0.44	7.48	7.6	90.03

compounds studied[17,18]. The uniformity of coating thickness of the sheet assured very precise measurements. Moreover there is no need for scraping and collecting the adsorbent, because it remains firmly attached to the solvent-resistant support. The adsorbed compound was eluted by immersing the entire cut-out piece in methanol. Notwithstanding the high eluting efficiency of methanol, the substances were not completely extracted from the adsorbent, but an appreciable and reproducible fraction was recovered as shown in Table I. Other workers have found that the analytical blanks are generally high and not reproducible owing to impurities contained in the silica gel and to silica particles which remain in suspension in the eluate[19].

In order to obtain constant and low values for the blanks, we removed areas equal in size to those occupied by the sample spots and at locations on the sheet with precisely equivalent R_F values[20]. Then the eluate was centrifuged as described earlier in order to eliminate the traces of finely divided silica gel[19]. Table I shows that low blanks were obtained in this manner. However, to reduce further any error resulting from this procedure, the corresponding standard compounds were simultaneously run through the procedure and were used to establish the content of unknown samples[21].

The results obtained by irradiating at 254 mμ a $5.52 \cdot 10^{-4}$ *M* *cis*-3-styrylpyridine solution in *n*-hexane are given in Table II. The data allow us to estimate the ratio of the two photocyclization products. This ratio is constant at 5.3 in favour of the 5,6-benzoisoquinoline at any time during the reaction. These data are in good agreement with the theoretical calculations.

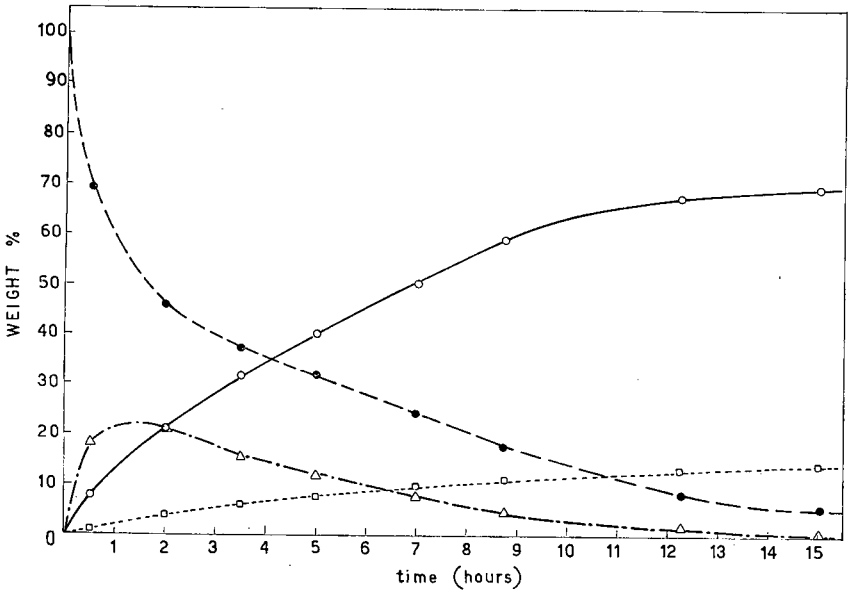

Fig. 3. Percentage of the reaction products as a function of time: □ 7,8-benzoquinoline; ○ 5,6-benzoisoquinoline; △ *trans*-3-styrylpyridine; ● *cis*-3-styrylpyridine.

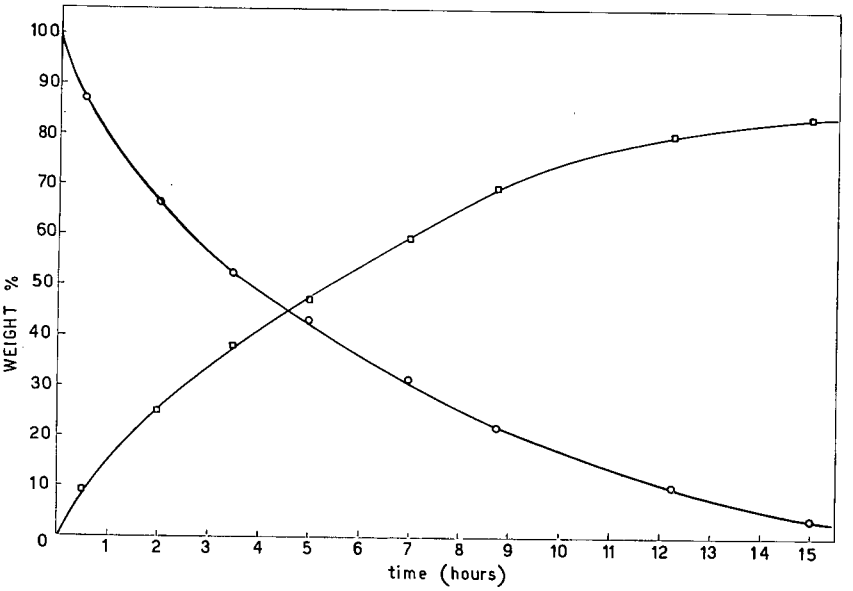

Fig. 4. Extent of photocyclization and photoisomerization: □ 7,8-benzoquinoline and 5,6-benzoisoquinoline, ○ *trans*- and *cis*-3-styrylpyridine.

The recovery of the compounds decreased with time (see Tables II and III). This is due to the increasing yield of the decomposition products which are often formed in many photochemical reactions.

The ratio of the *cis* and *trans* styrylpyridines is not constant with time of irradiation probably because of the fact that a photostationary composition of the two geometrical isomers is not reached owing to the competitive photocyclization of the *cis* compound[3].

The data are plotted in Figs. 3 and 4. On the basis of the plots in Fig. 4 it would appear that the total amount of the cyclization products formed is almost identical with the amount of the *cis–trans* styrylpyridines which disappears.

When the irradiation was carried out under the same conditions but using the 313 mμ, Hg line, the ratio of the two benzoquinolines was markedly increased in favour of the 5,6-benzoisoquinoline, as shown in Table III. The different ratio could indicate the formation of two intermediate dihydrobenzoquinolines in photochemical equilibrium with the reagent *cis*-3-styrylpyridine (see also ref. 16). Presumably the two possible forms of the derivatives have a different absorption spectrum and consequently a different photochemical equilibrium.

ACKNOWLEDGEMENTS

Our thanks are due to Miss R. LARGAIOLLI and Miss A. LUCCHIARI for their skilful technical assistance in performing this work.

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CHROM. 3635

THIN-LAYER CHROMATOGRAPHY OF RAT BILE AND URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF THE PESTICIDAL SYNERGIST OCTACHLORODIPROPYL ETHER

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SUMMARY

Chromatographic differences in rat bile and urine samples resulting from single intravenous administration of octachlorodipropyl ether were elaborated using Silica Gel DF-5 chromatoplates with toluene–acetic acid–water (10:10:1) as developer. Detection was accomplished by 2537 Å and 3660 Å ultraviolet as well as conc. sulfuric acid–*n*-butanol and silver nitrate–2 *N* alcoholic potassium hydroxide chromogenic reagents. Ten metabolites were detected in the bile and 5 in the urine. The relative rates of elimination of the major biliary and urinary metabolites were compared.

INTRODUCTION

Previously we reported on the elimination of pesticidal synergists (piperonyl butoxide and tropital)[1], ^{14}C-tropital[2] and related methylenedioxyphenyl derivatives, *e.g.*, safrole, isosafrole and dihydrosafrole[3] and their metabolites in rat bile and urine resulting from single intravenous administration of the above compounds.

It was found in these studies that the synergists piperonyl butoxide and tropital were altered chemically and although the rate of elimination was high, it did not reach a rapid peak and rapid subsequent decline but suggested *prolonged* elimination of the metabolites into the bile.

The purpose of this investigation was to follow by thin-layer chromatographic techniques the elimination of the synergist octachlorodipropyl ether** (I) and its metabolites in rat bile and urine following a single intravenous injection of the com-

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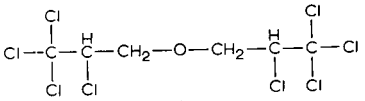
** Octachlorodipropyl ether (S-421) containing 1% stabilizer (epichlorohydrin) was obtained from Badische Anilin & Soda Fabrik, A-G, Ludwigshaven, Germany; b.p. 144–150°/1 mm.

pound and to compare its elimination with that of the methylenedioxyphenyl synergists studied above.



(I)

Octachlorodipropyl ether was first prepared by BECKE AND SPERBER[4] *via* the reaction of bis(chloromethoxy-methane) and trichloroethylene and has been shown to be a synergist for pyrethrum[5], pyrethrins, allethrin and cyclethrin[6,7], DDT[8], carbamate insecticides[9], including Sevin[9,10] and insecticidal organic phosphoric acid derivatives[11].

EXPERIMENTAL

Bile and urine sampling

Single intravenous injections of 0.05–0.09 ml of octachlorodipropyl ether were given to adult rats of the Sprague-Dawley strain averaging 350 g in weight. Bile samples were collected by fistula and urine samples by cannulation from each rat. Details on the handling of the animals, anesthesia, surgery, sample colllection and timing have been previously described[12]. At least three urine samples were collected, one before i.v. injection, a second at an appropriate interval after injection and a third sample at the termination of the bile collection. All samples were kept frozen until ready for analysis.

Preparation of the plates

Thin-layers (8 × 8 in.) 250 μ thick were prepared in the usual manner by mixing a slurry of Silica Gel DF-5* and water in a ratio of 30 g of absorbent to 72 ml water. They were air-dried for several hours, then oven-dried for half an hour at 75°, washed by ascending chromatography with chloroform–methanol (1:1) then oven activated at 75° for half an hour.

Solvent system

(a) Toluene–acetic acid–water (10:10:1).

Detecting reagents

Chromogenic agents. (a) Conc. sulfuric acid–*n*-butanol (15:85)[13]; (b) Silver nitrate–2 *N* alcoholic potassium hydroxide[14].

Plates were sprayed with 2 *N* methanolic potassium hydroxide, heated for 20–30 min at 120°, oversprayed with 1 % silver nitrate in 30 % nitric acid. Then the plates were exposed to unfiltered U.V. illumination for 15–20 min, or until spot intensity reached a maximum.

Radiation sources. (a) U.V. 3660 Å – Mineralight. Blak-Ray Model UVL-22**

(b) U.V. 2537 Å — Mineralight, Model UVS-11**.

* Obtained from Camag, Muttenz, Switzerland.

** Obtained from Allied Impex Corp., New York, N. Y.

Chromatography

Twenty microliters of all bile and urine samples were applied as half-inch streaks* on Silica Gel DF-5 plates and developed with toluene–acetic acid–water (10:10:1). Each developed plate was examined under visible light and 2537 Å and 3660 Å illumination, then photographed in color under 2537 Å illumination. Finally each plate was sprayed with the detecting reagent as described and photographed using equipment and procedures previously described[1].

RESULTS AND DISCUSSION

Chromatographic differences in bile and urine samples resulting from i.v. administration of octachlorodipropyl ether are summarized in Table I. Table I lists the

TABLE I

SUMMARY OF RAT BILE AND URINE R_F VALUES ON SILICA GEL DF-5 RESULTING FROM SINGLE INTRAVENOUS ADMINISTRATION OF OCTACHLORODIPROPYL ETHER

Solvent system: Toluene–acetic acid–water (10:10:1).
Detectors: (1) No spray; visible; (2) No spray; 2537Å; (3) Conc. sulfuric acid–butanol (15:85); (4) Conc. sulfuric acid–butanol (15:85)—3660Å; (5) Silver nitrate–2 *N* alcoholic potassium hydroxide.
Colors: Q = quench; B = blue; Bk = black; Bn = brown; G = green; Gr = grey; O = orange; V = violet; Y = yellow; T = tan.

Sample	$R_F \times 100$	*Detection agent*				
		1	*2*	*3*	*4*	*5*
Bile	15	—	—	Bk	Bk	—
	20	B-G	—	V	—	—
	30	—	—	—	—	Bn
	32	—	Q	—	—	—
	37	—	—	—	—	Bn
	43	—	—	—	—	Gr
	45*	Y-G	G	G	Q	—
	48	—	—	—	—	Gr
	60**	—	—	O-Bn	Y	—
	65	—	—	—	—	Gr
	78	—	—	T	Y-O	Gr
	81	—	—	T	Y-O	—
Urine	15	—	—	Bk	Bk	—
	30	—	—	—	—	T
	62	—	—	—	—	Gr
	68	—	—	—	—	Gr
	78	—	—	—	—	Gr
OCPE Std.	92	—	—	—	—	T

* A bile pigment.
** Cholic acid or its conjugate.

R_F value of each component obtained on Silica Gel DF-5 using toluene–acetic acid–water (10:10:1) as developer, and data regarding its characterization (means of detection and color). Other solvent systems screened for the resolution of biliary and

* Bile and urine samples were applied with a Radin-Pelids thin-layer sample streaker obtained from Applied Science Laboratories, State College, Pa., U.S.A.

TABLE II

RELATIVE CHANGES IN CONCENTRATION WITH TIME OF COMPONENTS APPEARING IN RAT BILE AFTER A SINGLE I.V. ADMINISTRATION OF OCTACHLORODIPROPYL ETHER*

Abbreviations: Bk = black; Bn = brown; G = green; O = orange; T = tan; V = violet; Gr = grey; tr = trace.

Detector: sulfuric acid–butanol (15:85)									*Detector: silver nitrate*							
$R_F \times 100$	*Spot color*	*Before i.v. injection*	*Color intensity hours after injection*					*Relative spot size*	$R_F \times 100$	*Spot color*	*Color intensity hours after injection*					*Relative spot size*
			0–1	*1–2*	*4–5*	*6–7*	*7–8*				*0–1*	*1–2*	*4–5*	*5–6*	*6–7*	
15	Bk	0	tr	2	4	4	4	4	30	Gr	0	6	2	1	1	1
20	V	0	1	3	3	4	4	1	37	Gr	3	4	4	4	3	4
**45	G	0	0	0	2	3	3	4	43	Gr	1	2	3	2	1	1
***60	O-Bn	2	2	3	3	3	3	4	48	Gr	0	3	3	2	1	1
18	T	trace	tr	2	2	2	2	1	65	Gr	0	1	1	tr	tr	1
81	T	0	0	1	1	1	1	1	78	Gr	0	tr	tr	tr	tr	1

* One i.v. dose of 0.092 ml of octachlorodipropyl ether administered to a 325 g male rat of Sprague-Dawley strain. Bile samples were collected during the indicated intervals. Spot intensities and sizes were estimated visually using arbitrary increments (0–4).

** A bile pigment.

*** Cholic acid or its conjugate.

TABLE III

RELATIVE CHANGES IN CONCENTRATION WITH TIME OF COMPONENTS APPEARING IN RAT URINE AFTER SINGLE I.V. ADMINISTRATION OF OCTACHLORODIPROPYL ETHER*

Spot intensities and sizes were estimated visually using arbitrary increments (0–4).

$R_F \times 100$	Spot** color	Color intensity					Relative spot size
		Before i.v. injection	Hours after i.v. injection				
			0–1	1–3	3–5	5–8	
30	T	0	1	4	3	2	4
62	Gr	0	0	tr	1	1	1
68	Gr	0	2	3	2	2	2
78	Gr	0	0	0	tr	tr	1

* One i.v. dose of 0.050 ml octachlorodipropyl ether administered to a 338 g male rat of Sprague-Dawley strain..

** As detected with the silver nitrate–2N alcoholic potassium hydroide reagent. Gr = grey; T = tan; tr = trace.

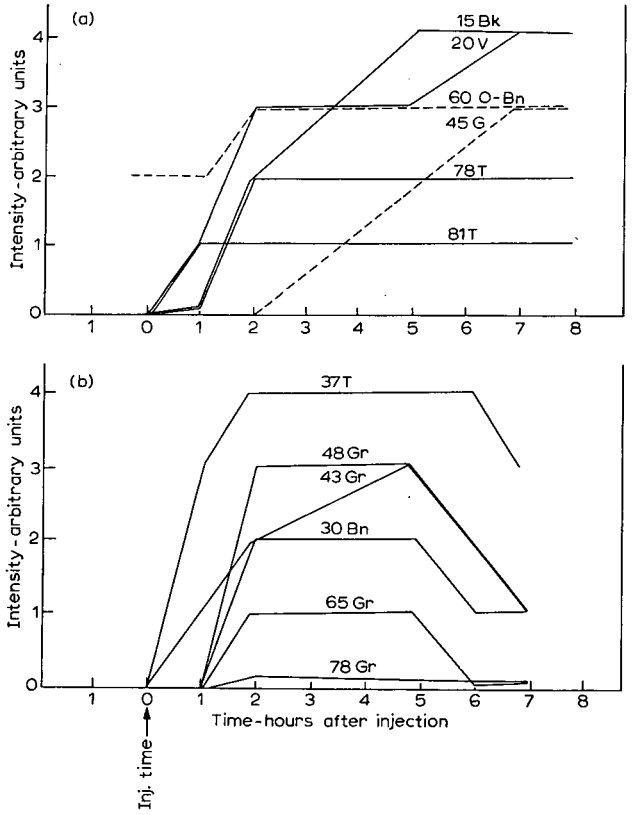


Fig. 1. Relationship of various bile components following intravenous administration of octachlorodipropyl ether. The data shown were taken from bile chromatograms developed with toluene–acetic acid–water (10:10:1). Concentrations were estimated visually as arbitrary degrees of intensity. Top and bottom figures illustrate biliary metabolites detected with (a) the sulfuric acid–butanol (15:85) and (b) silver nitrate–2 N alcoholic potassium hydroxide, respectively. Solid lines represent octachlorodipropyl ether metabolites and dotted lines depict bile components (*e.g.*, R_F 0.45 bile pigment, R_F 0.60 cholic acid or conjugate).

urinary metabolites, *e.g.*, (b) benzene–acetone (39:1) and (c) ethyl acetate–acetic acid–methanol (70:10:20) were found to be very much less effective than the toluene–acetic acid–water (10:10:1) system. Solvent system (c), however, effected the separation of two additional bile metabolites at R_F 0.40 and 0.84 (as revealed by the sulfuric acid–butanol treatment).

The sulfuric acid–butanol reagent detected four possible metabolites in the bile and one in the urine, while the silver nitrate reagent revealed five possible metabolites in the bile and four in the urine.

Table II depicts the change in concentration with time of each component in the bile detected by the sulfuric acid–butanol and silver nitrate reagents. Table III shows the change in concentration with time of each component in the urine after detection with silver nitrate. No detectable free octachlorodipropyl ether has been found in either bile or urine by the methods utilized above.

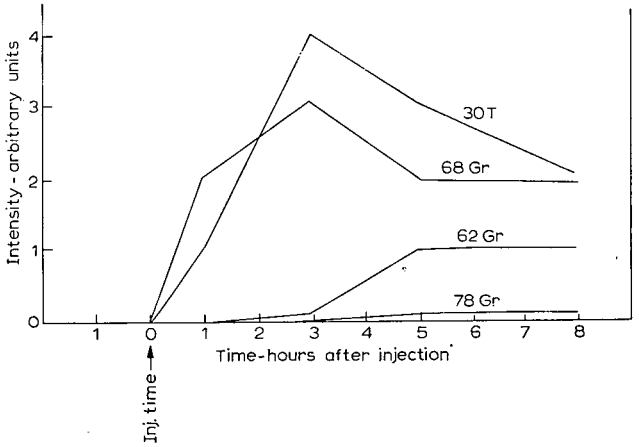


Fig. 2. Relationship of various urinary components following intravenous administration of octachlorodipropyl ether. The data shown were taken from urine chromatograms developed with toluene–acetic acid–water (10:10:1) and detected with the silver nitrate–2 *N* alcoholic potassium hydroxide reagent. Concentrations were estimated visually as arbitrary degrees of intensity.

Figs. 1 and 2 graphically depict the changes in concentration with time of components appearing in rat bile and urine respectively following a single i.v. administration of octachlorodipropyl ether. (The $R_F \times 100$ values and the spot colors correspond to those in Table I.) It can be seen from these figures that the rate of elimination of the biliary and urinary metabolites, although high (generally within 2 hours after administration of the drug), did not rapidly decline thereafter, but suggests slow prolonged elimination of the metabolites in the bile. This is analogous to the earlier experiments carried out with the methylenedioxyphenyl synergists piperonyl butoxide and tropital[1]. As suggested earlier, delayed elimination from the body of pesticides and other compounds coupled with inhibition of certain detoxification mechanisms could constitute a hazard to man when exposed to these compounds.

Although the metabolites found in the bile and urine chromatograms have not been identified thus far, it is of interest to speculate at this point as to their possible identity.

Fig. 3 depicts the possible routes of metabolism for octachlorodipropyl ether *via* successive steps of dehydrohalogenation, ether cleavage and hydrolysis. The possibility of hydroxy derivatives being formed and excreted in the urine as the corresponding glucuronide and/or sulfate conjugates exists, and is being currently explored. Similarly the possibility exists of formation of small fragment metabolites such as chloroform, methylene chloride or ethers (*e.g.* vinyl ether, etc.).

Fig. 4 depicts the possible formation of chlorinated acids and cyclic products formed from octachlorodipropyl ether *via* the sequences shown.

The reactivity of a number of linear and cyclic theoretical metabolites above

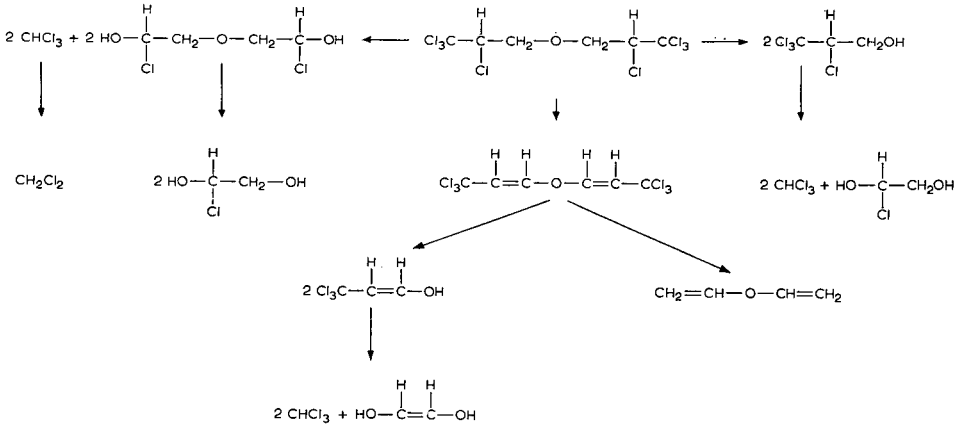


Fig. 3. Possible routes of metabolism of octachlorodipropyl ether to hydroxy derivatives, chlorinated hydrocarbons and ethers.

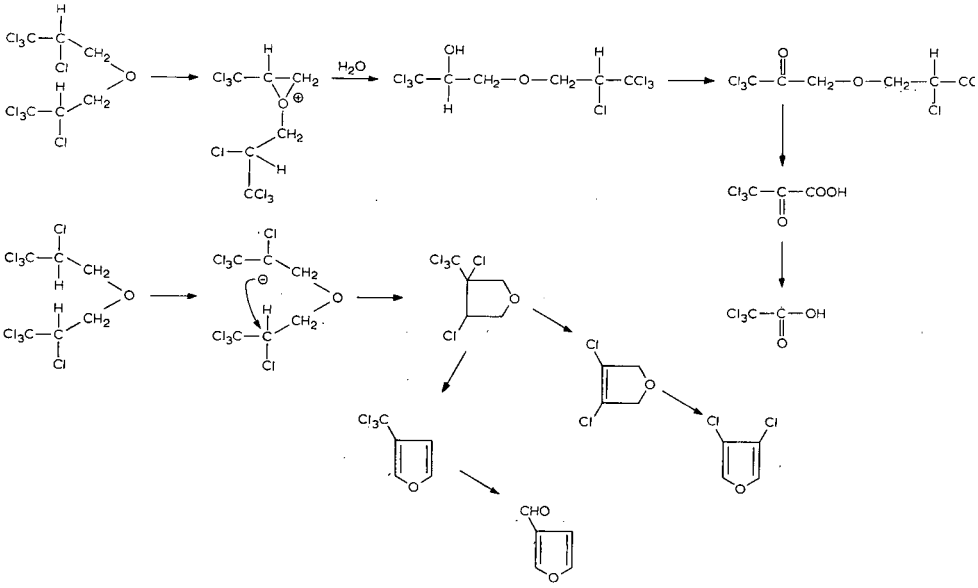


Fig. 4. Possible routes of metabolism of octachlorodipropyl ether to acids and cyclic derivatives.

in an aqueous milieu (*e.g.*, urine extract and the developing solvent for TLC, toluene–acetic acid–water (10:10:1)) could preclude their detection *per se*.

In this regard, gas chromatographic and gas chromatographic–mass spectrographic procedures are currently being explored for their utility in the separation and elaboration of the biliary and urinary metabolites of octachlorodipropyl ether following i.v. administration in the rat.

ACKNOWLEDGEMENT

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CHROM. 3655

SEPARATION OF SERUM ALBUMIN BY RADIAL CHROMATOGRAPHY ON NITROCELLULOSE MEMBRANES

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SUMMARY

Radial chromatography of serum proteins was investigated under various experimental conditions on nitrocellulose membranes. Development of about 15 μg/0.5 μl samples on Sartorius 11002 filters at pH 8.0–9.0, and with a salt concentration of up to 5 % NaCl at laboratory temperature (18–25°) was found most suitable for the small scale separation of serum albumin from the majority of other native or denatured serum proteins, within about 1 minute.

As reported earlier[1], serum proteins form two zones during radial chromatography on suitable nitrocellulose membranes at neutral and slightly alkaline pH. The inner zone seemed to correspond to the globulins, the outer to the albumin fraction.

The present communication deals in more detail with the finding mentioned above, by investigating the various conditions which have been assumed to enhance the desorption of proteins and by characterizing immunochemically the main protein zones formed during radial chromatography of serum. Some practical aspects, *e.g.*, a rapid separation of serum albumin and estimation of the albumin–globulin quotient, were also examined.

MATERIALS AND METHODS

Native human serum, gamma globulin and serum albumin from the Cohn's ethanolic fractionation (prepared in our Institute) as well as serum albumin denaturby heating a 1 % solution in Michaelis buffer[3] pH 8.5 at 100° (no precipitation occured) red were used as standard samples. Before radial chromatography the samples were diluted 1:1 by the given buffer and about 0.5 μl were applied to the origin wetted with buffer[1,5]. In some experiments serum and albumin were stained with bromophenol blue before application, to permit the convenient observation of the movement of the albumin fraction. Radial chromatography was performed on nitrocellulose membranes Synpor 3 and 6 (Chemapol, Prague) and Sartorius 11001, 11002, 11006 and 11011

* Director: Prof. J. HOŘEJŠÍ, M.D., DSc.

(Göttingen) as described earlier[1]. Strips 2 × 2.5 cm were used. The samples were developed continuously, the buffer being applied by a suitable capillary[1]. A series of isotonic Michaelis buffers[3] from pH 3.8 to pH 9.3 and veronal buffer pH 8.6[2], $\mu = 0.05$ were used to develop the chromatograms.

Both the outer and the inner protein zones were identified by micro-immunoelectrophoresis in agar gel[2]. Rabbit antihuman serum SEVAC-Prague (1:10,000) was used for immunoprecipitation. The outer zone of the chromatogram (presumably albumin stained by bromophenol blue) was cut out immediately after chromatography, divided into several pieces and placed at the start of the agar gel. A similar procedure was used to identify the inner zone on a parallel chromatogram. In that case, however, it was convenient to elute the proteins of the inner zone with 2 % Tween 60 in the Michaelis buffer, pH 8.5, containing 5 % NaCl, immediately after the first procedure mentioned above. The front of the second developing solution was visible without staining, so that it was also possible to cut out this zone immediately after developing, place it while still wet on the start of the agar gel and wet it with another drop of 2 % Tween 60 in Michaelis buffer, pH 8.5.

The chromatograms were stained in solutions of 0.01 % nigrosine or 0.05 % amidoblack 10 B in 3 % trichloroacetic acid for 10 min at laboratory temperature. Immunoelectrophoretic patterns were stained with a solution of 0.1 % amidoblack 10 B in an acetate buffer pH 3.5. Decolorisation of the background was carried out in 3 % acetic acid.

The determination of the albumin–globulin quotient A/G was attempted by radial chromatography of diluted human serum using the Michaelis buffer, pH 8.5. After staining the chromatogram in amidoblack 10 B for 10 min and decolorising the background, both zones were cut out close behind the colour edge and added to 1.5 ml of 0.1 *N* NaOH. The absorbancy of the solutions was measured at 630 nm using the Specol photocolorimeter.

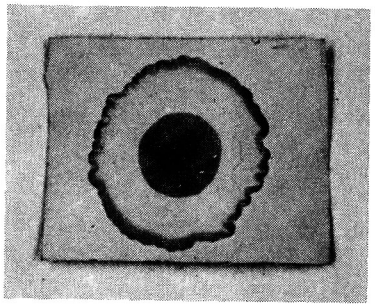
RESULTS AND DISCUSSION

The adsorption of serum proteins and especially of serum albumin on intact nitrocellulose membranes was found to change gradually at different pH values (*cf.* ref. 4). At pH 3.7 the serum proteins formed one uniform spot, with an area proportional to the protein concentration[1,5]. When chromatographed in a series of buffers with the pH value increasing between 3.8 and 9.3, the stepwise formation of two zones was observed beginning at about pH 6.5. The first fraction moved with the front of the developing buffer, while the second fraction remained adsorbed in the center of the circle (Fig. 1) even at pH 9.3. The best separations were achieved on Sartorius 11002 membranes at pH 8–9, preferably at 8.5.

As proved by immunoelectrophoresis, the outer area corresponded predominantly to serum albumin (Fig. 2), while the globulins remained mainly in the inner zone. However, a slight precipitation zone adhering to but not crossing the albumin zone was also observed (Fig. 2). Minute amounts of albumin in the inner zone as well as in the "free" area between the two zones were also found immunoelectrophoretically. Nevertheless, the chromatographic technique mentioned above permits a simple and rapid single-step micropreparation of crude serum albumin.

Comparative chromatographic experiments were also performed at pH 8.5

using isolated human gamma globulin and serum albumin instead of whole human serum. Albumin formed one main zone at the front but a slightly diffuse spot at the center was also observed. Gamma globulin only formed a single circular spot at the center of the chromatogram.



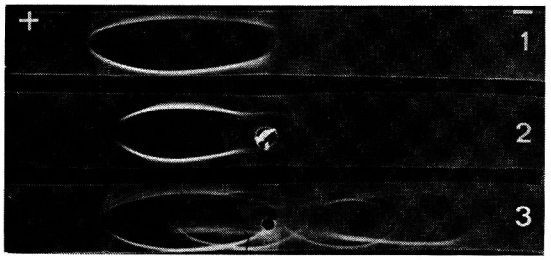


Fig. 1. Radial chromatography of human serum on Sartorius 11002 nitrocellulose membrane. Developed in Michaelis buffer[3] pH 8.5 for about 1 min. Outer diameter about 18 mm. The outer zone corresponds predominantly to serum albumin, the inner zone to the globulins.

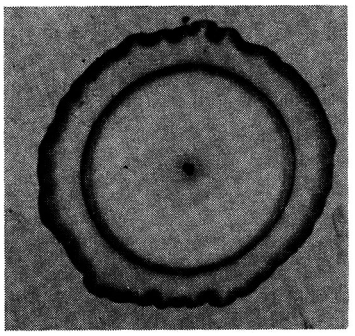
Fig. 2. Immunoelectrophoretical characterization of the "albumin" zone (see Fig. 1) in agar gel. 1 = Comparative run of human serum albumin; 2 = proteins of the outer zone (*cf.* Fig. 1); 3 = comparative run of human serum. Veronal buffer pH 8.6[2].

A similar result to that with gamma globulin was achieved with denatured serum albumin, and likewise with denatured and modified whole serum and globin[1]. The areas of the spots, formed as a result of circular chromatography of equal amounts of native and denatured serum, were significantly different: 100 % for native serum at pH 3.7, 30 % for the denatured one at pH 3.7 and 28 % for the denatured at pH 8.5. Apparently, denatured molecules and their aggregates are more tightly packed at the nitrocellulose surface or form higher multilayers. With regards to the firm, adsorption of denatured proteins and the globulins, even at pH's above 7, we are inclined to believe that it may be partly explained by the formation of nonpolar bonds between the lipophilic groups in the uncoiled parts of the protein molecules and the lipophilic groups of nitrocellulose. Changes in the conformation and molecular weight of the particles also seem to play an important role here[4]. Experimental work dealing with the above question is under investigation.

It seems to us that the inner zone observed on the chromatograms of isolated serum albumin probably consists of small amounts of "impurities", *e.g.* denatured albumin, globulins and also albumin polymers and aggregates. This was confirmed in separate experiments where the advancing front-zone was allowed to spread on to another nitrocellulose membrane (after having arranged a direct contact between them). No central spot corresponding to "impurities" was observed here. Consequently, membrane chromatography on nitrocellulose might be useful as a simple means for a rapid check on the quality of various albumin preparations.

Practically all firmly adsorbed proteins could be washed from the center by 2 % Tween 20, 40, 60 or 80 over the whole pH range tested (3.8–9.3) (Fig. 3). However, marked differences were observed depending on whether the 2 % Tween 60 had been dissolved in water or in Michaelis buffer, pH 8.5. When an unbuffered aqueous Tween

solution was used for the second elution, after chromatography of serum at pH 8.5, a part of the proteins still remained adsorbed at the central spot. It was possible to desorb them by adding 5 % NaCl to the solution of Tween (Fig. 4). Elution by different concentrations of Tween was not suitable for fractionation, since it has the property of replacing chromatography with an "all or none" elution effect on proteins. A series of various reagents and conditions, *e.g.* development by 8 *M* urea in water or with increasing concentrations of 0.9–20 % NaCl, as well as the use of extreme pH values on the acid and alkaline sides, were not successful in attempts to desorb the proteins or to achieve their further fractionation. This fact was probably due to the denaturation of the proteins under those conditions.



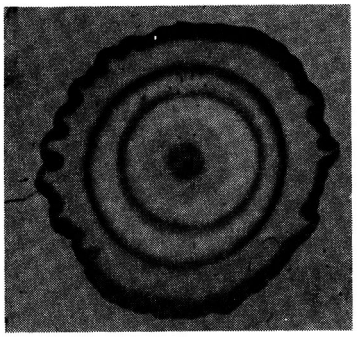


Fig. 3. Double development of human serum. First development was identical to that in Fig. 1. For the second development 2 % Tween 60 in Michaelis buffer, pH 8.5, was used.

Fig. 4. Triple development of human serum. First development as in Fig. 1; the second was made by 2% Tween 60 in water, the third by 2% Tween in 5% NaCl.

However, it was found possible to use the separation of albumin from other serum proteins on nitrocellulose membranes, at slightly alkaline pH, for a rapid and simple determination of the albumin–globulin quotient in a clinical laboratory. In preliminary experiments we found the A/G quotient of normal human serum to be 2.0, which is in good agreement with frequently encountered data.

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THE PAPER CHROMATOGRAPHY OF SOME ISOMERIC MONOSUBSTITUTED PHENOLS. III.

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SUMMARY

In a continuation of previous work, the R_F' values have been determined for a number of isomeric phenols in three polar and three non-polar solvent systems. The use of ceric ammonium nitrate as a spot-locating agent for phenols has been continued and found to be of much value. Some correlation has been found between the R_F' value of some alkoxyphenols and the molecular weight of the substituent, especially with the *meta* and *para* isomers. With the *ortho* isomers internal bonding effects upset this relationship. Mineral spirits has been found to be a good indicator of the degree of polarity of a molecule.

INTRODUCTION

The studies in the first two papers in this series were carried out on isomeric monosubstituted phenols.[1,2] The solvent systems used were water, toluene saturated with water and mineral spirits saturated with water. A solution of ceric ammonium nitrate was found to be a satisfactory spot-locating agent in most cases. In some cases, a correlation was found between R_F' values and the molecular weight of the substituent groups.

The effects of the orientation of substituents in the various solvents were studied. The findings confirm the work of many others in showing an "*ortho* effect" in which the *ortho* isomer has the highest R_F value in an isomeric series, followed by the *meta* and finally the *para* isomer. This "*ortho* effect" has also been shown with disubstituted phenols in which the 2,6-isomer has the highest R_F value in an isomeric series.[3-5]

The work presented in this paper is an extension of the previous data. Three additional solvent systems have been developed in order to obtain additional information. Isomeric monosubstituted phenols are still the main groups studied but some additional groups or single phenols have been included to provide additional information.

EXPERIMENTAL

Reagents

The mobile solvents used were divided into polar and non-polar solvents. The polar solvents were water, water–dioxane–acetic acid (75:24:1, v/v/v), and water–methanol–acetic acid (75:24:1, v/v/v). The non-polar solvents were benzene saturated with water, toluene saturated with water and mineral spirits saturated with water. The mineral spirits had a Kauri-butanol value of 37–38 and an aniline point of 56–59°. The spot-locating agent was a ceric ammonium nitrate solution which has been described earlier.[1]

Procedure

The non-polar solvents were kept saturated with water by the presence of a lower water phase in the chromatographic chamber. The chambers were all lined with paper extending into the liquid to maintain a saturated atmosphere at 25°. The ascending method of development was used with Whatman No. 1 paper. The papers were allowed to equilibrate in the chambers for 20 min before being immersed in the mobile solvents. This was omitted, however, when examining volatile materials such as the isopropylphenols. Solvent travel was 10 cm, requiring 30–60 min depending on the solvent used.

The paper sheets were removed from the chamber and sprayed immediately with the ceric ammonium nitrate reagent. When methanol was present in the solvent, the papers were dried before spraying. After spraying, any visible spots were marked, including a few in which the material bleached the reagent white. The papers were washed with warm tap water to remove excess reagent and then air-dried and the remaining spots marked. Nearly all the spots fade slightly on aging but are permanent enough to provide a good visual record. The R_F' values reported are the ratio of the spot front to the solvent front.[6]

RESULTS AND DISCUSSION

Previous work in this series has shown that water, toluene saturated with water and mineral spirits saturated with water are useful mobile solvents for substituted phenols. This has been found to be true in the present data. The three additional solvent systems examined have also been found to be of value. Care must be used with the two solvent systems B and C (Table I) since on prolonged usage the composition changes due to loss of volatile components. These solutions are renewed once a week or oftener if used frequently. Changing composition is not a problem with the other four solvents since three of them are pure liquids and the fourth is quite non-volatile. These solutions have been used over several months duration with no change observed in R_F' values obtained.

The data obtained for a number of phenols in the six solvent systems are shown in Table I. As in the previous work, a ceric ammonium nitrate solution was found to be a useful spot-locating agent. In a few cases, the spots were light in color and required larger samples than normally used but the majority of phenols gave easily discernable spots. In one case (*m*-hydroxybenzophenone) we were unable to locate any spot when solvent system B was used. Both *p*-nitrobenzenediazonium fluoborate

TABLE I

R_F' VALUES OF SOME ISOMERIC MONOSUBSTITUTED PHENOLS

Solvent systems: (A) water; (B) water–dioxane–acetic acid (75:24:1, v/v/v); (C) water–methanol–acetic acid (75:24:1, v/v/v); (D) benzene saturated with water; (E) toluene saturated with water; (F) mineral spirits saturated with water.

Phenol	R_F' *Value at 25°*					
	A	*B*	*C*	*D*	*E*	*F*
o-Cyanophenol	0.75	0.92	0.83	0.72	0.64	0.07[a]
m-Cyanophenol	0.73	0.90	0.81	0.88	0.79	0.10
p-Cyanophenol	0.71	0.88	0.79	0.73	0.61	0.00
o-Hydroxycinnamic acid	0.53	0.85	0.68	0.06	0.06	0.00
m-Hydroxycinnamic acid	0.50	0.84	0.65	0.05	0.04	0.00
p-Hydroxycinnamic acid	0.46	0.80	0.57	0.04	0.00	0.00
o-Hydroxyphenylacetic acid	0.84	0.91	0.86	1.00	1.00	0.58[a]
m-Hydroxyphenylacetic acid	0.86	0.93	0.86	1.00	0.97	0.15[a]
p-Hydroxyphenylacetic acid	0.80	0.89	0.84	1.00	0.96	0.11[a]
o-Isopropylphenol	0.88[b]	0.97[b]	0.85[b]	1.00	1.00	0.98
m-Isopropylphenol	0.75	0.95	0.80	1.00	1.00	0.94
p-Isopropylphenol	0.68	0.90	0.76	1.00	1.00	0.92
o-Hydroxybenzophenone	0.00	0.51[a]	0.62	1.00	1.00	1.00
m-Hydroxybenzophenone	0.00	—	0.63	1.00	1.00	0.20
p-Hydroxybenzophenone	0.00	0.86[a]	0.67	1.00	1.00	0.00
o,o'-Dihydroxybenzophenone	0.51[a]	0.88	0.70[a]	1.00	1.00	0.99
p,p'-Dihydroxybenzophenone	0.41[a]	0.90	0.56	0.11	0.06[a]	0.00
o-Hydroxydiphenylmethane	0.60[a]	0.83[a]	0.76[a]	1.00	1.00	0.95
p-Hydroxydiphenylmethane	0.44[a]	0.80[a]	0.66[a]	1.00	1.00	0.85
o,o'-Dihydroxydiphenylmethane	0.64[a]	0.90	0.75	1.00	0.98	0.38
p,p'-Dihydroxydiphenylmethane	0.60	0.90	0.75	0.97	0.56	0.00
o-tert.-Amylphenol	0.00	0.49[a]	0.00	1.00	1.00	1.00
p-tert.-Amylphenol	0.00	0.00	0.00	1.00	1.00	0.89
o,o'-Dihydroxyazobenzene	0.00	0.67[a]	0.00	1.00	1.00	0.99
p,p'-Dihydroxyazobenzene	0.00	0.64[a]	0.00	0.00	0.00	0.00
p,p'-Dihydroxyazoxybenzene	0.12[a]	0.65	0.20[a]	0.00	0.08[a]	0.00
p-Hydroxyazobenzene	0.24[a]	0.72[a]	0.36[a]	1.00	1.00	0.47[a]
m-Hydroxymandelic acid	0.91	0.94	0.93	0.33	0.18	0.00
p-Hydroxymandelic acid	0.90	0.92	0.90	0.27	0.15	0.00
m-Hydroxydiphenylamine	0.50[a]	0.84	0.61[a]	1.00	1.00	0.31[a]
p-Hydroxydiphenylamine	0.54[a]	0.86	0.63[a]	1.00	1.00	0.30[a]
m-Butoxyphenol	0.66[a]	0.83	0.72	1.00	1.00	0.91
p-Butoxyphenol	0.00	0.82	0.00	1.00	1.00	0.00
p-Hydroxyphenylsulfone	0.75	0.94	0.78	0.93[a]	0.54	0.00
p,p'-Dihydroxydiphenyl ether	0.00	0.82	0.67[a]	0.00	0.00	0.00

(*continued on p. 271*)

TABLE I (*continued*)

Phenol	R_F' *Value at 25°*					
	A	*B*	*C*	*D*	*E*	*F*
Diphenolic acid	0.70	0.91	0.83	0.08	0.12[a]	0.00
1-Hydroxyfluorene	0.19[a]	0.65	0.34[a]	1.00	1.00	0.67
2-Hydroxyfluorene	0.14[a]	0.66	0.30[a]	1.00	1.00	0.46[a]
3-Hydroxyfluorene	0.17[a]	0.73	0.34[a]	1.00	1.00	0.56
9-Hydroxyfluorene	0.22	0.77	0.45[a]	1.00	1.00	0.69
1-Hydroxyfluorenone	0.00	0.74[a]	0.28[a]	1.00	1.00	0.92
2-Hydroxyfluorenone	0.00	0.69[a]	0.27[a]	0.83	0.83	0.00
4-Hydroxyfluorenone	0.00	0.72[a]	0.27[a]	0.93	0.93	0.00
2-Hydroxypyridine	0.88	0.88	0.86	0.10[a]	0.06[a]	0.00
3-Hydroxypyridine	0.86	0.85	0.87	0.12[a]	0.08[a]	0.00
4-Hydroxypyridine	0.81	0.81	0.84	0.00	0.00	0.00
4-Hydroxyhydrindene	0.68	0.82	0.72	1.00	1.00	0.84
5-Hydroxyhydrindene	0.56	0.73	0.64	1.00	1.00	0.80
2-Hydroxyquinoline	0.52[a]	0.79	0.65	0.74	0.64	0.00
4-Hydroxyquinoline	0.71	0.83	0.78	0.00	0.00	0.00
5-Hydroxyquinoline	0.77	0.83	0.78	0.27[a]	0.20[a]	0.00
6-Hydroxyquinoline	0.77	0.80	0.74	0.00	0.00	0.00
7-Hydroxyquinoline	0.77	0.81	0.80	0.00	0.00	0.00
8-Hydroxyquinoline	0.81	0.84	0.78	1.00	1.00	0.94
2-Hydroxy-1,4-Naphthoquinone	0.00	0.83[a]	0.61[a]	0.87	0.91[a]	0.21[a]
8-Hydroxy-1,4-Naphthoquinone	0.00	0.81[a]	0.59[a]	1.00	1.00	0.93
p-Nitrosophenol	0.75	0.89	0.82	0.32[a]	0.23[a]	0.00
p-tert.-Octylphenol	0.00	0.00	0.00	1.00	1.00	0.90
p-Dodecylphenol	0.00	0.00	0.00	1.00	1.00	0.95

[a] Spot streaks.
[b] Vaporizes rapidly.

and alkaline potassium permanganate were ineffective in this case. The reasons for our failure to locate this spot in this solvent are not known at the present time.

It has been observed previously that less readily ionized phenols have greater R_F' values with mineral spirits and toluene than with water.[2] A number of additional cases have been observed in the current data including all of the isopropylphenols, both hydroxydiphenylmethanes, the *tert.*-amyl phenols and a number of others as shown in Table I. A number of *ortho* isomers are also in this group. The use of the additional solvent systems gives supporting evidence in most cases. The phenols that have higher R_F' values with mineral spirits and toluene also have higher R_F' values with benzene. Many of these values are greater than the R_F' values found not only with water but also the other two polar solvents. This is especially true with benzene and toluene since they are both extremely strong solvents with non-polar phenols. The presence of *ortho* substituted phenols in this group shows the reduced polarity caused by intramolecular bonding effects. The fact that the hydroxyfluorenes, hydroxy-

fluorenones and hydroxyhydrindenes are in this group shows that these compounds are not readily ionized and thus are non-polar in nature.

The use of benzene as a non-polar solvent has shown that it gives the highest R_F' values of any of this group. This result is not unexpected since benzene is slightly higher in various eluotropic series than is toluene.[7,8] In the three polar solvent systems, the solvent water–dioxane–acetic acid gives much higher R_F' values than the other two. This is somewhat unexpected since both water and methanol are high in the eluotropic series, both having greater dielectric constants than dioxane. In this case the dioxane should act as a non-polar diluent but apparently it acts as a strong organic solvent.

The solvent system water–methanol–acetic acid results in R_F' values that are higher than those obtained with water alone. Apparently the methanol (as does the dioxane) acts as an organic solvent at least partially independent of the polarity of the phenol or the dielectric constant of the solvent.

The effect of the substituent group orientation on R_F' values for four of the solvent systems is shown in Figs. 1–4. The data for the solvent systems water–methanol–acetic acid and benzene were not included since they closely resemble other solvent systems. In Figs. 1 and 2, the order of the substituents as the R_F' values decrease is quite similar in most cases. The use of dioxane–acetic acid along with water results in an overall increase in R_F' values as can be seen from comparing Fig. 1 with Fig. 2. As observed previously the mechanism involved in the case of the polar solvents is primarily adsorption.

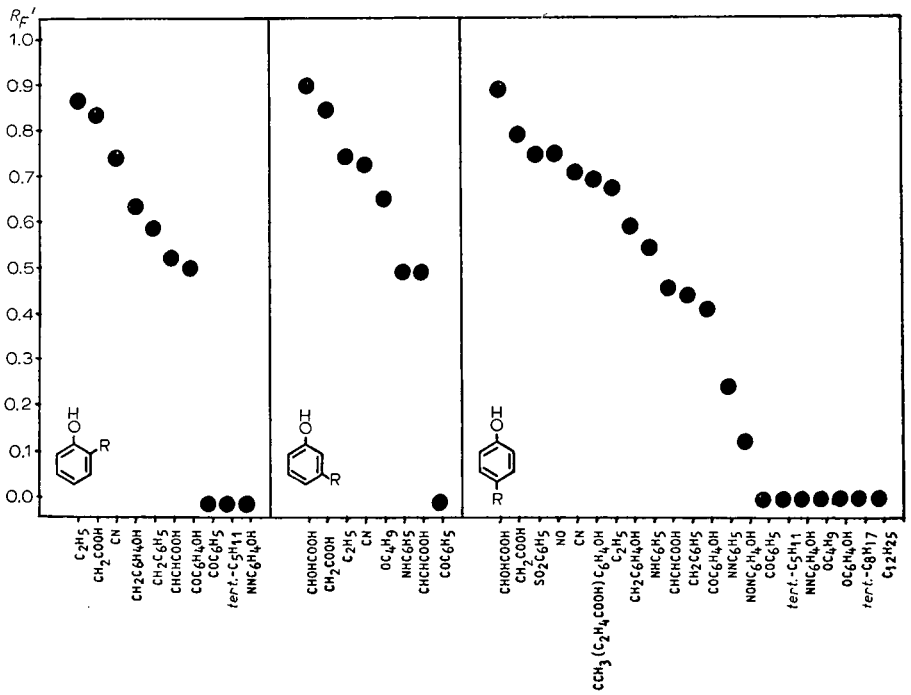


Fig. 1. Effect of orientation of substituents on the R_F' value with water as solvent.

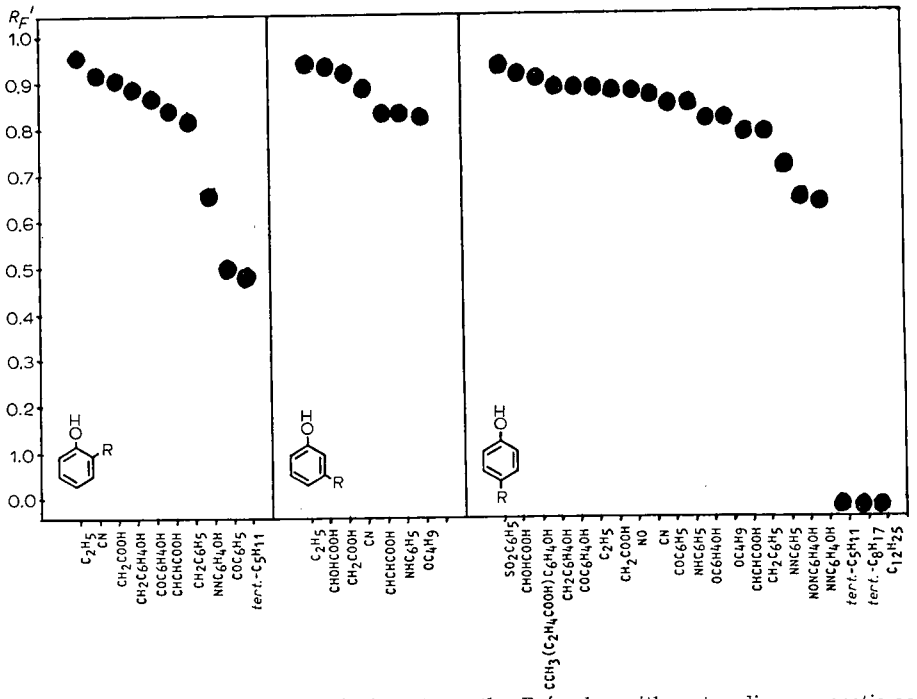


Fig. 2. Effect of orientation of substituents on the R_F' value with water–dioxane–acetic acid as solvent.

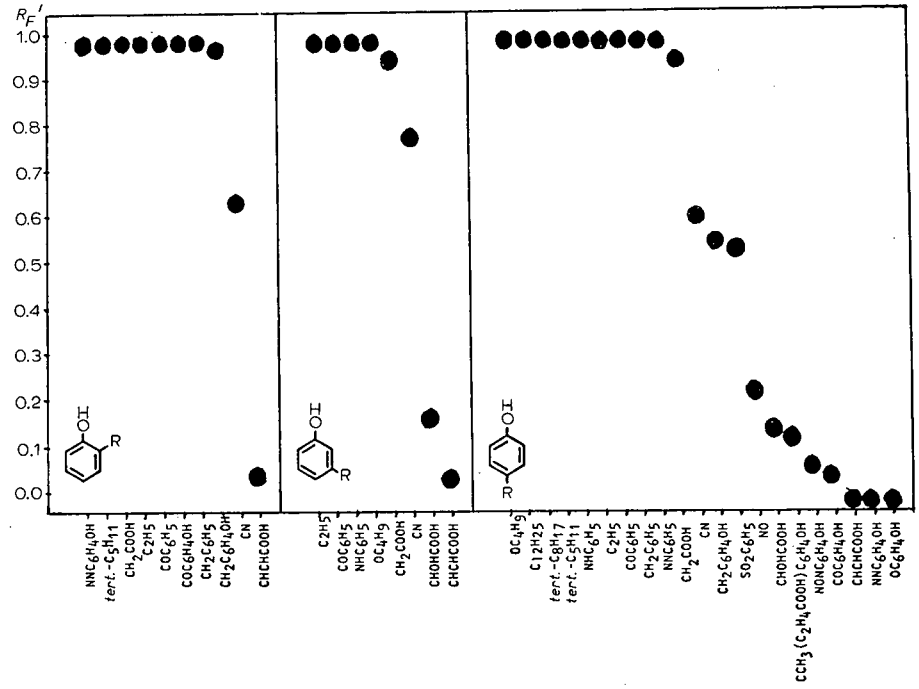


Fig. 3. Effect of orientation of substituents on the R_F' value with toluene as solvent.

Figs. 3 and 4 show the order of the substituents as the R_F' values decrease to be in nearly the same order with the non-polar solvents. In this case, the mechanism involved is partition and again the great differences between adsorption and partition can be seen by comparing the order of the substituents in the polar solvent systems with the order in the non-polar systems.

Since data are now available for the *meta* and *para* isomers of three alkoxyphenols, a comparison was made of the R_F' values and the molecular weights of the substituents in water and mineral spirits. The data for the two lower members of the series were taken from Part II of this paper. The results of this comparison are shown in Fig. 5. Although not included in Fig. 5, the *ortho* isomers of the two lower members

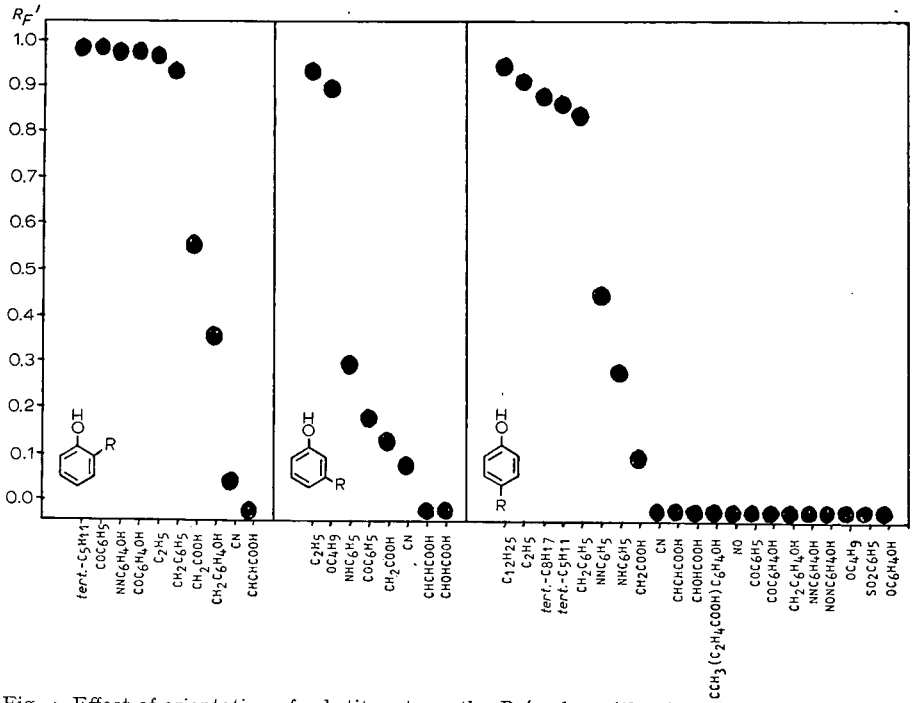


Fig. 4. Effect of orientation of substituents on the R_F' value with mineral spirits as solvent.

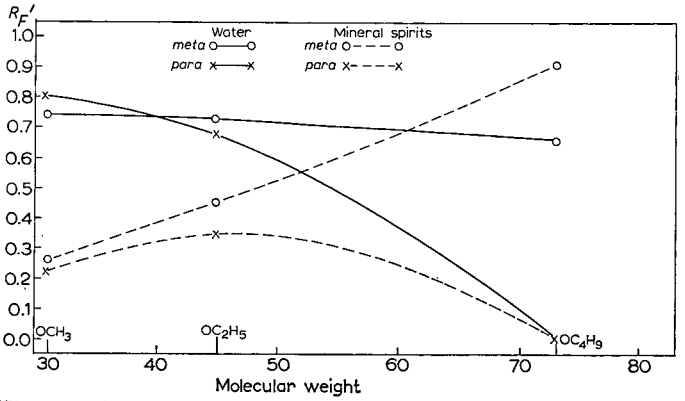


Fig. 5. R_F' values in water and mineral spirits *vs.* molecular weight of alkoxy side chain.

of the series show considerable deviation from the patterns shown by the *meta* and *para* isomers. The *ortho* isomers were not included since only two points would be available. Based on these values, it would not be possible to predict even the approximate R_F' value for *o*-butoxyphenol. Apparently the intramolecular bonding in the *ortho* isomers upsets the molecular weight–R_F' relationship.

The data for the isopropylphenols observed in the current work have been compared with those for the *n*-propylphenols in Part II. With both water and mineral spirits, the isopropylphenols had higher R_F' values than did the *n*-propylphenols. This was especially pronounced with water as the solvent.

Comparisons were made between the R_F' values for *p*-hydroxyazobenzene and *p,p'*-dihydroxyazobenzene to determine the effect of the extra hydroxyl. As might be expected, the extra hydroxyl (especially in the *para* position) lowers the R_F' values rather drastically.

A similar comparison was made between *o*- and *p*-hydroxybenzophenone and *o,o'*- and *p,p'*-dihydroxybenzophenone. In this case, the results were quite different. The extra hydroxyl group in the *ortho* position increased the R_F' values in the polar solvents but had little effect with the non-polar solvents. The extra *para* hydroxyl group increased the R_F' values in the polar solvents (except when methanol was present) but drastically reduced the R_F' values with the non-polar solvents. The *ortho* substituted benzophenones can readily form intramolecular hydrogen bonds and thus reduce the R_F' values in polar solvents. This is particularly true for the benzophenone containing only one hydroxyl group in the *ortho* position. The addition of a second *ortho* hydroxyl allows the molecule to become more polar and thus more susceptible to the effects of polar solvents.

A comparison of *o*- and *p*-hydroxydiphenylmethane with *o,o'*- and *p,p'*-dihydroxydiphenylmethane appears to give results analogous to the benzophenones. However, the variations in R_F' values are not as severe possibly because of weaker internal bonding.

Some comparisons have been made for the R_F' values of four sets of isomeric phenols containing an acidic substituent. These include the hydroxyphenylacetic acids, the hydroxybenzoic acids (data in Part I), the hydroxycinnamic acids and the hydroxymandelic acids. The results as shown in Fig. 6 indicate that the R_F' values are

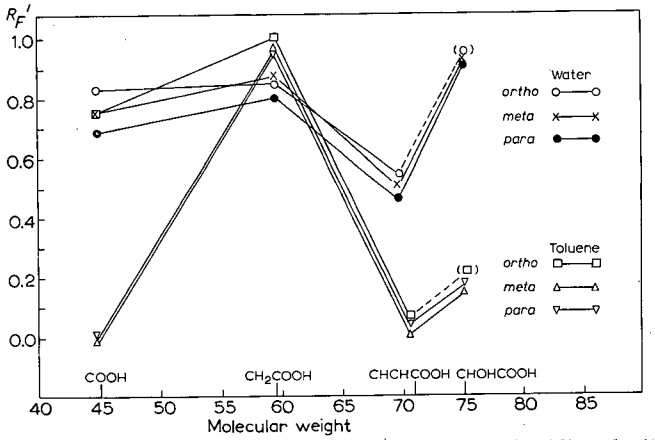


Fig. 6. R_F' value of phenols *vs.* molecular weight of acidic substituent.

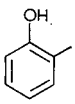
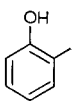
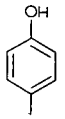
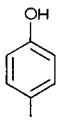
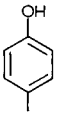
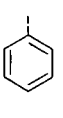
not a direct function of the molecular weights. With water as the solvent, the unsaturation present in the cinnamic acid substituent has a pronounced lowering effect on the R_F' values. The three saturated substituents have R_F' values that appear to be nearly proportional to the molecular weights. This does not hold true in toluene, however. The data do enable us to make a prediction for the R_F' values of *o*-hydroxymandelic acid, which is not available. Thus, based on Fig. 6, we can estimate the R_F' value with water to be between 0.91 and 0.95 and with toluene to be between 0.18 and 0.25. The estimated values are shown in parentheses in Fig. 6 and are connected with dotted lines.

The effect of inserting a methylene group between the ring and the carboxyl group can be seen by comparing the data for the hydroxybenzoic acids and the hydroxyphenylacetic acids. In the three solvents common to both sets of isomers it is seen that insertion of a methylene group between the ring and the carboxyl group causes an increase in R_F' values. This increase is quite drastic with the non-polar solvents, indicating that the polarity of the molecule has been reduced because of the longer chain length.

The data for a number of compounds containing two phenyl groups connected by some intermediate group have been examined. In a case where the two phenyl groups are identical, any variations in R_F' values must come from the connecting group. These data are shown in Table II. In the four separate groupings in this table,

TABLE II

R_F' VALUES OF SOME PHENOLS HAVING TWO PHENYL GROUPS WITH VARIABLE CONNECTING GROUPS

Phenyl	*Phenyl*	*Connecting group*	*Solvent*					
			A	*B*	*C*	*D*	*E*	*F*
		$-CH_2-$	0.64[a]	0.90	0.75	1.00	0.98	0.38
		$-\overset{\overset{O}{\|\|}}{C}-$	0.51[a]	0.88	0.70[a]	1.00	1.00	0.99
		$-N=N-$	0.00	0.67[a]	0.00	1.00	1.00	0.99
		$H_3C-\overset{\|}{C}-CH_2CH_2COOH$	0.70	0.91	0.83	0.08	0.12[a]	0.00
		$-\overset{\|}{C}H_2-$	0.60	0.90	0.75	0.97	0.56	0.00
		$-\overset{\overset{O}{\|\|}}{C}-$	0.41[a]	0.90	0.56	0.11	0.06[a]	0.00
		$-NON-$	0.12[a]	0.65	0.20	0.00	0.08[a]	0.00
		$-O-$	0.00	0.82	0.67[a]	0.00	0.00	0.00
		$-N=N-$	0.00	0.64[a]	0.09	0.00	0.00	0.00
		$-CH_2-$	0.60[a]	0.83[a]	0.76[a]	1.00	1.00	0.95
		$-\overset{\overset{O}{\|\|}}{C}-$	0.00	0.51[a]	0.62	1.00	1.00	1.00
		$-SO_2-$	0.75	0.94	0.78	0.93	0.54	0.00
		$-NH-$	0.54[a]	0.86	0.63[a]	1.00	1.00	0.30[a]
		$-CH_2-$	0.44[a]	0.80[a]	0.66[a]	1.00	1.00	0.85
		$-N=N-$	0.24[a]	0.72[a]	0.36[a]	1.00	1.00	0.47[a]
		$-\overset{\overset{O}{\|\|}}{C}-$	0.00	0.86[a]	0.67	1.00	1.00	1.00

[a] Spot streaks.

it is seen that when the connecting group is $-CH_2-$ the highest R_F' values are usually found. This appears to be independent of the fact that either one or both phenyl groups contain a hydroxyl substituent. When mineral spirits is the solvent, the -C(O)- group gives higher R_F' values than the $-CH_2-$ because of reduced polarity from intramolecular bonding. The relationship of polarity to R_F' value with mineral spirits is discussed later.

In the event that both phenyl groups contain a hydroxyl substituent, the -C(O)- connecting group usually has the second highest R_F' values. However, when only one phenyl group contains a hydroxyl substituent, the R_F' value for the connecting group -C(O)- becomes 0.00 with water and in most other solvents is equal to or greater than the R_F' value for the $-CH_2-$ connecting group. This is because of the reduced polarity of the molecule caused by the loss of one of the hydroxyl groups.

The three non-polar solvents show generally high R_F' values independently of the connecting group except where both phenyl groups have *para* hydroxyl substituents. In this case the molecules are more polar since the opportunity for internal bonding is reduced.

When the connecting group is the azo-nitrogen configuration, some interesting comparisons can be made. With the three polar solvents, the R_F' value is higher when only one of the phenyl groups has a hydroxyl substituent. When both phenyl groups contain a hydroxyl substituent, the polarity is reduced possibly as a result of a resonance structure facilitated by the azo connecting group. This lowers the R_F' values. When only one hydroxyl is present, a resonance structure would be less likely to form resulting in higher polarity and higher R_F' values with polar solvents.

With the three non-polar solvents and an azo connecting group, when both phenyl groups have *para* hydroxyls, the R_F' values are 0.00. However, in both other cases with non-polar solvents the R_F' values are very high. Apparently the R_F' values with the non-polar solvents are not dependent on the solubility of the molecule but rather the polarity. When both phenyl groups have *para* hydroxyls, the polarity is high, resulting in low R_F' values with non-polar solvents. When both phenyl groups have *ortho* hydroxyls, internal bonding reduces the polarity, resulting in higher R_F' values with non-polar solvents.

The effect of adding oxygen to the azo connecting group can be seen by comparing the data for the azo and azoxy diphenols. There is little or no effect in the non-polar solvents but with polar solvents the R_F' values are higher. The addition of the oxygen would reduce the resonance effect and thus increase the polarity of the molecule, resulting in a higher R_F' value.

For purposes of obtaining additional information, a number of phenols have been examined that are not strictly classified as monosubstituted phenols. These include a number of isomeric groups such as hydroxynaphthoquinones, hydroxyfluorenones, hydroxyfluorenes, hydroxyhydrindenes, hydroxyquinolines and hydroxypyridines.

With only a few exceptions this group has relatively high R_F' values with the non-polar solvents, especially the highly aromatic solvents. This is because of the relatively large size of the molecules involved which tends to overshadow polar effects and thus results in compounds that are largely non-polar in composition. The major exceptions to this are the hydroxypyridines, which are relatively small in size and thus more likely to be affected by polarity. This is supported by the fact that all of the hydroxypyridines have high R_F' values with polar solvents.

Polar effects cannot be completely ignored, however, since the hydroxyquinolines show otherwise. The four isomeric hydroxyquinolines that have the least opportunity for intramolecular bonding are also seen to have low R_F' values with all of the non-polar solvents. These are the 4,5,6- and 7-hydroxyquinolines. If the 3-hydroxyquinoline were available, it too would presumably fall in this category. Conversely, the 2- and 8-hydroxyquinolines, which can easily form bonds because of their steric positions, have high R_F' values with non-polar solvents. The 8-hydroxyquinoline appears to bond more readily than the 2-isomer since its R_F' value is greater, especially with the mixed aromatic–aliphatic solvent mineral spirits.

A similar effect is seen with both the naphthoquinones and the fluorenones. In these examples the 1-hydroxyfluorenone and the 8-hydroxy-1,4-naphthoquinone are most tightly bonded internally as shown by the high R_F' value with non-polar solvents and especially with mineral spirits. With polar solvents 8-hydroxy-1,4-naphthoquinone, as expected, has a slightly lower R_F' value than does the 2-isomer. This is not the case with 1-hydroxyfluorenone, however.

From a survey of the data in Table I, as well as that in Parts I and II of this paper, it appears that in most cases mineral spirits may be used as a good indicator of the degree of intramolecular bonding. When the bonding is strong the R_F' value with mineral spirits is high since the molecule tends to be non-polar. For this reason most *para* isomers have low R_F' values with mineral spirits except when the substituent is either a non-hydroxyl containing aromatic or a saturated aliphatic group. In these cases, the R_F' values are high because of the similarity in structure between the solvent and the substituent.

It is believed that bonding effects are weak with the hydroxyfluorenes and the hydroxyhydrindenes. Since 9-hydroxyfluorene has the highest R_F' value, it may bond with either of the aromatic rings to reduce its polarity. However, this reduction in polarity seems insufficient to cause low R_F' values with the polar solvents. This same fact appear to be true with 4-hydroxyhydrindene. With the fluorenes the compound in which the second greatest amount of bonding could occur would be that with the hydroxyl in the 1-position. In this position the hydroxyl could bond with a hydrogen from the 9-position. As before, however, this bond is so weak that the R_F' value with non-polar solvents is not affected.

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PAPER ELECTROPHORESIS OF MONOHYDRIC ALCOHOLS AND HYDROXY ACIDS

SEPARATION AS XANTHATES

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SUMMARY

The paper electrophoresis of the potassium salts of xanthates derived from monohydric alcohols is described. Primary and secondary alcohols of different structural types are characterised by the relative mobilities of the corresponding xanthates using sodium hydrogen carbonate as electrolyte. The method proved to be useful for the detection and identification of alcohols present as impurities in solvents.

Mono-carboxylic hydroxy acids yield carboxy-alkyl xanthates which are readily separable from the respective free hydroxy acid anions also present in the reaction mixtures. Both anions may be detected simultaneously on pherograms.

Conditions are described under which the unstable xanthates derived from tertiary alcohols may be separated electrophoretically from their reaction mixtures and detected on the pherograms.

A method is described for the identification of the alkoxy groups of esters using the xanthate reaction. The acyl groups of the esters may be identified simultaneously.

The electrophoretic mobilities of some common sulphur-containing inorganic anions, including trithiocarbonate and trithiopercarbonate, were determined in sodium hydrogen carbonate electrolyte. The detection of the inorganic anions, the xanthates and some organic acid anions present on pherograms is described.

The xanthate reaction has been applied in various ways for the identification of alcohols[1,2–4], and paper and thin-layer chromatography of potassium alkyl xanthates as means of separating and identifying alcohols have been the subject of several reports[5–8]. BERBALK[9] has described the paper electrophoresis of xanthates derived from a number of mono- and di-hydric alcohols and has measured their anionic mobilities in a Veronal buffer.

A selection of monohydric alcohols representative of several classes has now been characterised by paper electrophoresis of the corresponding xanthates using sodium hydrogen carbonate as electrolyte. These include terpene alcohols, hydroxy acids, some carbohydrate derivatives containing a single free hydroxyl group and the glycol ether solvents known commercially as Cellosolves and Carbitols. The homologous series of aliphatic derivatives studied by BERBALK are also included and their

behaviour in sodium hydrogen carbonate and other electrolytes is reported for purposes of comparison.

Xanthates derived from tertiary alcohols are unstable in aqueous media and it is probably for this reason that the chemical literature contains no reference to the chromatography of these derivatives. We have found that some tertiary xanthate preparations partially survive the more rapid process of paper electrophoresis and are separable from reaction mixtures as elongated spots or streaks.

The scope of the method has been extended to the direct identification of the alkoxy groups of esters. The acyl groups of the esters may be identified simultaneously on pherograms as the corresponding acid anions.

Xanthate preparations usually contain inorganic anions[10]. These include sulphide, thiosulphate and trithiocarbonate, which either form simultaneously with the xanthates or result from their decomposition, especially in aqueous media. The electrophoretic mobilities of these and other sulphur-containing anions in sodium hydrogen carbonate are recorded.

EXPERIMENTAL

Materials

Commercial samples of alcohols, hydroxy acids, esters and other reagents were used, in most cases, without further purification, but samples of tertiary alcohols were subjected to treatment for the removal of impurities, mainly primary or secondary alcohols.

tert.-Butyl alcohol was purified by repeated fractional crystallisation from its own melt[11].

tert.-Amyl alcohol and *dl*-α-terpineol were each purified by allowing the commercial sample to react in pyridine solution with toluene-*p*-sulphonyl chloride at room temperature for 3 h during which primary and secondary alcohols present in the samples as impurities were selectively tosylated[12]. The reaction mixtures were poured on to crushed ice and excess toluene-*p*-sulphonyl chloride was allowed to hydrolyse. After the addition of cold hydrochloric acid, the aqueous media were extracted with ether and the extracts washed with $NaHCO_3$ solution and dried over anhydrous magnesium sulphate. The ether was removed and the residues distilled under reduced pressure to obtain the pure tertiary alcohols.

The carbohydrate derivatives were each prepared according to published procedures[13–17].

Sodium salts of sulphate, sulphite, sulphide and thiosulphate were of reagent grade. A solution of tetrathionate was prepared by oxidising sodium thiosulphate with iodine. Sodium trithiocarbonate was prepared according to the directions of INGRAM AND TOMS[18].

Electrolytes

(a) Sodium hydrogen carbonate solution (0.1 *M*, pH 8.4), hereinafter abbreviated to "$NaHCO_3$ solution".
(b) Sodium carbonate solution (0.1 *M*, pH 11.2).
(c) Sodium hydroxide solution (0.1 *M*).
(d) Acetic acid–sodium acetate buffer (0.1 *M* with respect to acetate, pH 6.0).
(e) Veronal buffer (0.1 *M*, pH 9.8).

Spray reagents

(a) Ammonium molybdate (3 % w/v) in 2 % sulphuric acid (v/v)[6,19].
(b) Copper sulphate solution (7.5 %)[6].
(c) Mercuric chloride solution (6 %).
(d) Chromium trioxide–permanganate–sulphuric acid. The preparation and use of this reagent as a spray for papers after electrophoresis have been described[20].
(e) Iodine (0.5 %) in 1 % potassium iodide solution.
(f) Sodium nitroprusside (10 %) in water.
(g) Bromphenol blue reagent. The indicator (0.04 g) was dissolved in 95 % ethyl alcohol (100 ml) and the solution adjusted to pH 6.7 by adding dilute alkali[21].

Apparatus

Paper electrophoresis was conducted in the enclosed strip apparatus described previously[22] using Whatman No. 4 paper in strips 13.5 × 61 cm, with 45 cm underpressure and cooled. The circulation of ice-water through the coils of the cooling-plate at a rate of about 2 l/min maintained the temperature at the surface of the paper at 4° during electrophoresis in all electrolytes.

In some experiments, mains water at 24° was circulated and maintained the temperature of the paper at 26°. Circulating water at 48° maintained the paper at 50°.

Procedure

Potassium alkyl xanthates were prepared from the alcohols by the method of WHITMORE AND SIMPSON[23]. The alcohols (0.2 ml) were each shaken for a few minutes in a small tube at room temperature with powdered potassium hydroxide (0.3 g) and carbon disulphide (0.5 ml). Xanthation of the more viscous alcohols was facilitated by triturating reaction mixtures with a glass rod.

Solid alcohols, including the carbohydrate derivatives, and hydroxy acids, solid and liquid, were converted to the corresponding xanthates using similar quantities of reactants in solution in dimethyl sulphoxide (1 ml).

The reaction products were normally subjected to paper electrophoresis after dilution with water to give solutions which were 0.02 to 0.06 *M* with respect to the amount of alcohol used, but more concentrated solutions of some derivatives were required to facilitate their subsequent detection. Reaction mixtures of carbohydrate derivatives were diluted to 0.1–0.15 *M* concentration, and the *n*-decyl compound to 0.2 *M*. The reaction mixtures of tertiary alcohols were applied directly to papers without dilution.

The alkoxy groups of esters were converted to xanthates by shaking each ester (0.2 ml) in a stoppered tube for 5 min at 50° with a mixture of potassium hydroxide (0.3 g) and dimethyl sulphoxide (1 ml), adding carbon disulphide (0.5 ml) to the mixture after cooling and then stirring for 3–5 min. Reaction mixtures were applied to the papers after dilution with water (30 ml).

Samples of 0.5 μl of xanthate solutions were transferred by means of a platinum loop to papers impregnated with electrolyte and equilibrated for 15 min by enclosure within the apparatus. (The concentrated reaction mixtures of tertiary alcohols were viscous and unknown volumes considerably in excess of 0.5 μl were transferred to papers with the loop.)

Inorganic salts were applied, usually as 0.1 *M* solutions in water.

Caffeine was used as the marker for zero migration and rates of migration of the test compounds were calculated relative to that of nitrobenzene-*p*-sulphonate applied to the same paper (M_N values[20]).

Under the standard conditions, electrophoresis was allowed to proceed in $NaHCO_3$ solution at 4° for 1 h at about 21 V/cm. (Nitrobenzene-*p*-sulphonate moved approximately 8 cm under the standard conditions.) In other experiments, conditions were varied with respect to pH, duration and temperature.

The papers were dried in the oven at 100°. Caffeine, nitrobenzene-*p*-sulphonate, the xanthates and some of the inorganic anions present were located as dark blue spots under a Hanovia "Chromatolite" ultraviolet lamp. Papers were treated with one of the spray reagents to confirm the location and, in some cases, the identities of the spots.

Tertiary xanthates were subjected to electrophoresis at 4° for 15–20 min and the papers partially dried at 100° for 2–3 min. They were sprayed, without delay, with molybdate or other reagent.

Test for thiosulphate

The test described by FEIGL[24] for thiosulphate was adapted in the following way to its detection after electrophoretic separation.

Dried alkaline papers were sprayed uniformly with formic acid solution (0.3 *M*) until phenolphthalein (applied away from spots of test compounds) was just decolourised and remained colourless after re-drying the papers in the oven. The papers were then sprayed lightly and evenly with mercuric chloride solution, followed, without drying, by the bromphenol blue reagent. Yellow spots appeared on a blue ground where sulphuric acid, generated by reaction of the mercuric ion with thiosulphate, had liberated free formic acid from the sodium salt formed on the paper during the initial spray treatment.

RESULTS AND DISCUSSION

The results of the electrophoresis of 36 xanthates under the standard conditions are given in Table I. The derivatives are classified according to the type of alcohol from which they were prepared.

The effect of pH

The $NaHCO_3$ solution was selected as the standard electrolyte because it was found to promote more rapid separations of xanthates than the sodium carbonate or sodium hydroxide electrolytes. The derivatives were stable, however, at the higher values of pH and the same relative order of mobilities prevailed. Some of the inorganic ions gave less distinct spots at higher pH.

Some decomposition of most xanthates was evident in acidic electrolytes at pH 5 in the form of streaking, but in acetate buffer at pH 6 the derivatives were stable and migrated as compact spots.

The effect of temperature

Electrophoresis was conducted at 4° in $NaHCO_3$ solution for maximum stabilization of the xanthates which, with two or three exceptions, gave compact, almost

TABLE I

RELATIVE RATES OF MIGRATION OF XANTHATE IONS

Compounds were detected after paper electrophoresis in sodium hydrogen carbonate (0.1 *M*) at 21 V/cm and 4° for 1 h. For each parent compound, the mol. wt. of the derived xanthate ion is included.

Parent compound	*Derived ion*	
	$M_N \times 100$[a]	*Mol. wt.*
Aliphatic alcohols		
1. Methyl	143	107
2. Ethyl	122	121
3. *n*-Propyl	108	135
4. Isopropyl	111	135
5. *n*-Butyl	100	149
6. Isobutyl	97	149
7. *sec.*-Butyl	100	149
8. *n*-Amyl	88	163
9. Isoamyl	88	163
10. *n*-Hexyl	84	177
11. Cyclohexyl	86	175
12. *n*-Heptyl	76	191
13. *n*-Octyl[b]	63	205
14. Capryl (octan-2-ol)	65	205
15. *n*-Nonyl[b]	54	219
16. *n*-Decyl[c]	49	233
17. Benzyl	87	183
Unsaturated alcohols		
18. Allyl	118	133
19. α-Furfuryl	98	173
Glycol ethers		
20. 2-Methoxy-ethanol (Methyl Cellosolve)	111	151
21. 2-Ethoxy-ethanol (Ethyl Cellosolve)	101	165
22. 2-(2-Methoxyethoxy)-ethanol (Methyl Carbitol)	90	195
23. 2-(2-Ethoxyethoxy)-ethanol (Ethyl Carbitol)	83	209
Terpene alcohols		
24. Geraniol	66	229
25. Isopulegol	70	229
26. Menthol	70	231
Carbohydrate derivatives		
27. Methyl 2,3,6-tri-O-methyl-α-D-glucopyranoside	66	311
28. 2,3 : 4,6-Di-O-isopropylidene-L-sorbose	63	335
29. 1,2 : 5,6-Di-O-isopropylidene-D-glucose	63	335
30. Methyl 3-O-methyl-4,6-O-benzylidene-β-D-idoside	58	371
31. 1,2 : 5,6-Di-O-cyclohexylidene-D-glucose	54	415
Hydroxy-acids		
32. Glycollate	182 (129)[d]	150
33. Lactate	157 (108)	164
34. β-Hydroxy-butyrate	150 (104)	178
35. Mandelate	136 (87)	226
36. 2,4 : 3,5-Di-O-methylene-D-gluconate	116 (77)	294

[a] M_N values[20] express mobilities relative to the nitrobenzene-*p*-sulphonate ion, which moved approx. 8 cm.

[b] *n*-Octyl and *n*-nonyl xanthates gave somewhat elongated spots.

[c] The value of $M_N \times 100$ given for *n*-decyl xanthate corresponds to the head of a streak observed for this derivative.

[d] Values of $M_N \times 100$ in parentheses are relative mobilities of the respective parent acid anions.

circular spots. Most derivatives migrated satisfactorily with little or no sign of decomposition at 26°, but elongation of some spots was noted after experiments conducted at 50° in $NaHCO_3$ solution.

The derivatives suffered little decomposition during heating to dry the papers after electrophoresis and many survived prolonged heating at 100°.

In most cases, unreacted parent alcohols volatilised from the papers during drying but some of the less volatile appeared as non-migrating spots with some spray reagents. Unreacted hydroxy acids were detected as additional anionic spots. The presence of dimethyl sulphoxide in a preparation resulted in the appearance of a relatively large spot ($M_N = 0$) with the chromium trioxide–permanganate–sulphuric acid reagent.

Detection of xanthates, organic acids and inorganic anions after electrophoresis

Xanthates on paper chromatograms fluoresce in ultraviolet light[5] and the fluorescence of trithiocarbonate, trithiopercarbonate and the xanthates was strong enough to enable detection of the ions in the small quantities (about 0.02 μmole) needed for the electrophoretic method.

Grote's reagent has been used successfully for the location of xanthates on paper chromatograms[5] but it lacked the sensivity required and was abandoned in the present work in favour of the ammonium molybdate[6,19] and copper sulphate[6] reagents. With the exception of the tertiary xanthates described below, the molybdate reagent gave pink spots which slowly (20–30 min) changed to blue[25]. Thiosulphate, tetrathionate, trithiocarbonate and trithiopercarbonate gave blue reactions developing slowly. The copper reagent gave stable, bright yellow or yellow-brown spots with xanthates.

Salts of other heavy metals were also tried. Silver nitrate solutions, either ammoniacal[7] or acidic, gave brown or black spots but were only moderately sensitive. Concentrated aqueous solutions of cobalt and nickel salts[6] gave only weak spots with most xanthates in the concentrations used here. Mercuric chloride, however, was almost as sensitive to xanthates as the copper reagent. When it was sprayed on to dried papers impregnated with alkali metal carbonates, brown basic mercuric carbonate precipitated on the cellulose fibres and xanthates appeared immediately as stable white spots. The reagent is not specific and was used for some inorganic ions, especially sulphate, otherwise difficult to detect. The organic acid anions, succinate, benzoate and acetate formed as by-products in experiments with esters, were also located with mercuric chloride, but for these and the inorganic ions it was less sensitive and the presence of 0.1–0.2 μmole of each was required for satisfactory results.

Chromium trioxide–permanganate–sulphuric acid reacted with xanthates to give immediate yellow spots on a pink background. The papers were stored between glass plates and the spots became white within 7–10 min, the background simultaneously changing to brown. A permanent record of the experiment was obtained in the manner previously described by FRAHN AND MILLS[20]. The reagent was very sensitive to xanthates, although not specific, and less than 1 μg of methyl xanthate per sq. cm of spot area was easily located. The reagent was sensitive to oxalate and hydroxy acid anions and was used for their detection in preference to the mercuric chloride reagent. Thiosulphate and tetrathionate gave strong reactions, but sulphite, trithiocarbonate and trithiopercarbonate reacted weakly.

The iodine reagent caused immediate oxidation of xanthates on paper, forming white spots on a brown ground. It was very sensitive, though non-specific, and provided an alternative means of detecting thiosulphate, sulphite, trithiocarbonate and trithiopercarbonate.

Nitroprusside did not react with xanthates and was used to confirm the presence of sulphide in some xanthate preparations following its electrophoretic separation. Nitroprusside gave a strong violet reaction quickly fading to pink with trithiocarbonate and an immediate pink colour with trithiopercarbonate. Thiosulphate gave a stable pale blue spot which formed slowly.

Xanthates from primary and secondary alcohols

The xanthate reaction is quickly and easily performed and provides a convenient means of converting primary and secondary alcohols to stable charged species suitable for electrophoresis.

The present study was undertaken as a basis for the development of a simple method of enumerating the hydroxyl groups of polyhydroxy compounds without reference to their molecular weights and it was found during the preparatory work that electrophoretic mobility provided a useful guide for identifying a wide range of mono-functional xanthates. The inverse relationships which exist between their relative mobilities and molecular weights are illustrated graphically in Fig. 1, prepared from data contained in Table I.

The points plotted for the lower members of the aliphatic series lie on or near a smooth curve—the upper portion of curve A. The break which occurs in the curve beyond the *n*-heptyl derivative (compound 12) is probably due to an increasing tendency for derivatives with longer carbon chains to form ionic micelles large enough to suffer retardation by the cellulose support. The streak observed for the *n*-decyl

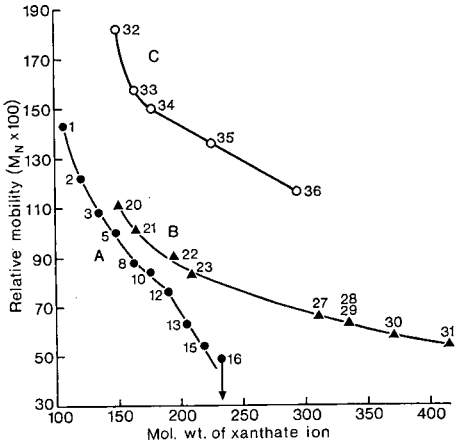

Fig. 1. Relationships between mol. wt. and relative mobility of xanthate ions derived from structurally different types of monohydric alcohols. Numerals correspond to parent compounds as listed in Table I. Curve A: xanthates of normal aliphatic primary alcohols. The arrow appended to the point plotted for the *n*-decyl derivative (16) indicates the streak that was observed in this case. Curve B: xanthates of alcohols containing ether, cyclic acetal, cyclic ketal and glycosidic bonds. Curve C: xanthates derived from hydroxy acids.

derivative may have been the result of the same effect in exaggerated form. Curve A, extrapolated from the points for members immediately lower in the series, passes through a point corresponding approximately to the mid-point of the streak for the *n*-decyl ion.

The set of curves comprising Fig. 1 indicate that the xanthates may be divided into three distinct groups or families. Curve A corresponds to members of the aliphatic series, as noted above, curve B to derivatives containing oxygen in ether, glycoside, cyclic acetal or cyclic ketal bonds (those of the glycol ethers and carbohydrates) and curve C to xanthates derived from hydroxy acids. The data for the unsaturated derivatives (compounds 18 and 19) appear to fit curve B.

The electrophoretic method as applied to homologous alkyl xanthates gives results which are complementary to those of the chromatographic methods inasmuch as the sequence in which the members separate is inverted[7].

The results for members of the aliphatic series (Table I) agree generally with those of BERBALK[9], an exception being the case of the *n*-propyl derivative, which occupies an anomalous position in the series as described by BERBALK. Using Schleicher and Schüll 2043b paper impregnated with Veronal buffer at pH 9.8, he found *n*-propyl xanthate to have a mobility identical with that of the *n*-butyl derivative. Under our standard conditions of electrophoresis these derivatives separated readily after being applied to the paper as a mixture. The same separation was observed when the other alkaline electrolytes, including Veronal buffer, were substituted for $NaHCO_3$ solution and when Schleicher and Schüll paper replaced Whatman No. 4.

BERBALK reported similar mobilities for isobutyl and isoamyl xanthates and for the benzyl and *n*-propyl derivatives, but we found these pairs to separate quickly in all alkaline electrolytes. These discrepancies are not the effects of differences in temperature, unless these were very great, because experiments in which the temperature was maintained at 26° and at 50°, using Veronal buffer, did not yield results significantly different from those in $NaHCO_3$ solution at 4°.

Xanthates from tertiary alcohols

It is well known that stable xanthates are obtained in comparable yields from aliphatic primary and secondary alcohols, and similar reactivities are displayed by primary and secondary hydroxyl functions contained in compounds of more complex structure. Xanthation of the isomeric di-O-isopropylidene derivatives of L-sorbose and D-glucose containing, respectively, a primary and a secondary hydroxyl group, proceeded with approximately equal facility to yield stable derivatives.

In contrast, xanthate ions derived from tertiary alcohols are unstable in aqueous media. WHITMORE AND LIEBER[1] considered that their rapid hydrolysis involved regeneration of the parent alcohols with the formation of sulphide. But it seems that hydrolysis of at least some of these derivatives is by no means instantaneous. For example, an aqueous solution of potassium *tert.*-amyl xanthate has been used for the preparation of the insoluble indium salt[27].

The survival of some tertiary xanthates in an aqueous medium was demonstrated by electrophoresis in $NaHCO_3$ solution. The preparation of these derivatives occasionally calls for special methods[28,29], but the usual conditions suffice in many cases[1,26,27]. For our experiments, xanthates were prepared in the normal way from pure *tert.*-butyl and *tert.*-amyl alcohols, *dl*-α-terpineol and *cis*-terpin. They were

applied to papers in concentrated form and subjected to electrophoresis for brief periods as described above. The application of molybdate to the partially dried papers revealed the tertiary xanthates as elongated anionic spots or streaks. The leading edges of the streaks often lagged a little behind spots of the respective isomeric primary or secondary derivatives run in other lanes. Streaking probably resulted from the combined effects of decomposition of the migrating ions and overloading of the papers with concentrated reaction mixtures. The tertiary xanthates reacted in a normal way with each spray reagent except molybdate. The streaks were coloured blue instantly upon the application of molybdate in contrast to the behaviour of other xanthates which formed pink spots slowly changing to blue[25]. The effect was not due to the liberation of sulphide from the decomposing ions because no response was obtained to nitroprusside in separate tests. Nor was the effect due to the application of concentrated reaction mixtures to the papers, because xanthates prepared from primary and secondary alcohols and applied to papers in undiluted form were subsequently located with molybdate as large spots, typically pink in colour.

cis-Terpin, containing two tertiary hydroxyl groups, gave two distinct streaks, the leading edges of which had values of $M_N \times 100$ of 68 and 115. These probably corresponded to the mono- and di-xanthates, respectively, of the parent glycol.

When the products of reaction of the pure tertiary alcohols were diluted and subjected to electrophoresis, no xanthates were detected on the resulting pherograms, but under the same conditions, most commercial samples of the same tertiary alcohols gave rise to faint pink spots with molybdate. Primary or secondary alcohols present as impurities proved to be responsible for these spots. Gas–liquid chromatography of the commercial samples confirmed the presence of impurities as supernumerary peaks accompanying the main peaks.

Carboxy-alkyl xanthates

The mono-carboxylic hydroxy acids listed in Table I all reacted rapidly to give deep red or red-brown equilibrium mixtures containing the corresponding xanthates in high proportions when dimethyl sulphoxide was used as a solvent for the reactants. In the absence of the solvent the reactions were generally sluggish.

Unreacted hydroxy acids were always present in the diluted reaction mixtures and migrated as anions in alkaline electrolytes. The mobilities of the derived xanthates, each containing two negative charges, were correspondingly greater and promoted their rapid separation from the free hydroxy acid anions.

The carboxy-alkyl xanthates reacted in a manner typical of xanthates with spray reagents applied to papers after electrophoresis. Free hydroxy acids, with the exception of the dimethylene gluconate, were detected simultaneously with the xanthates by using chromium trioxide–permanganate–sulphuric acid. The gluconate was located only with mercuric chloride. Relative mobilities of the hydroxy acid anions are included in Table I together with those of the respective xanthates. Each hydroxy acid is thus characterised by a set of two values.

The corresponding derivatives of the dicarboxylic hydroxy acids, malic and tartaric, were not detected. Although the S-ethyl ester of 1,2-dicarboxyethyl xanthic acid derived from malic acid is known[30], it seems that the free xanthic acid anion is unstable. Similarly, citric acid, containing a tertiary hydroxyl group, does not form a stable xanthate.

Attempts to demonstrate the formation of a xanthate from pyruvate, which might be expected to react in the enolic form, were unsuccessful. Two components were, however, readily separated from reaction mixtures, one of which ($M_N \times 100 = 134$) was pyruvate. The other ($M_N \times 100 = 146$) proved to be 2-hydroxy-2-methyl-4-oxo-glutarate, a dimer of pyruvate, formed by aldol-type condensation of pyruvate in the alkaline reaction medium[31]. Pyruvate and its dimer reacted strongly with chromium trioxide–permanganate–sulphuric acid and with iodine, but slowly with the latter. The dimer reacted with other reagents used for the detection of xanthates but not in a typical manner. It formed a blue spot slowly with molybdate and a faint green spot with copper.

The dimer is an impurity in most commercial and laboratory-prepared samples of sodium pyruvate[32]. It is biochemically interesting in that it is a potent inhibitor of the tricarboxylic acid cycle and has been shown to be specific for the enzyme α-keto-glutaric oxidase[33].

Detection of alcohols as impurities in solvents

It has been shown[5] that 0.1% of methyl alcohol contained in ethyl alcohol is easily detectable by xanthation followed by paper chromatography and we have used the electrophoretic method for similar demonstrations.

Experiments to show that some commercial samples of *tert.*-butyl and *tert.*-amyl alcohols contain the isomeric primary or secondary alcohols as impurities have already been described. α-Terpineol was commonly found to contain an alcoholic impurity which reacted readily to form a xanthate with a mobility identical with that of geraniol.

Ethylene glycol and diethylene glycol (Digol) were similarly shown to be impurities of some samples of the glycol ethers, Methyl and Ethyl Carbitol (compounds 22 and 23). The di-xanthates of the glycols, having relative mobilities ($M_N \times 100$) of 156 and 140, respectively, separated easily from each other and from the xanthates of the glycol ethers.

Further proof of the identities of the impurities was obtained by subjecting the crude glycol ether samples to xanthation conditions in which carbon disulphide was present in quantities insufficient to allow the reactions to go to completion. Mixtures of the mono- and di-xanthates of each of the glycol impurities were then formed together with the xanthates of the glycol ethers. The mono-xanthates of ethylene glycol and Digol have relative mobilities ($M_N \times 100$) of 112 and 97, respectively, and separations of all five xanthates contained in each reaction mixture were achieved under the standard conditions of electrophoresis.

It may be noted that the mobility of the derivative obtained from ethylene glycol by BERBALK[9] corresponds to that of the mono-xanthate.

Identification of alkoxy groups of esters

The procedure given in the Experimental section was adopted after it was found that, under the ordinary conditions of the xanthate reaction, alkaline hydrolysis of esters occurred to a limited extent only, although no special precautions were taken against the entry of moisture. Insufficient amounts of the component alcohols were liberated to form xanthates in quantities easily detectable after electrophoresis. The behaviour of diethyl oxalate was exceptional in this respect, however. It reacted vigorously with the solid potassium hydroxide and the mixture was found to

TABLE II

RELATIVE RATES OF MIGRATION OF XANTHATE IONS DERIVED FROM ESTERS

The standard conditions of electrophoresis given in Table I were used.

Esters	$M_N \times 100$	
Dimethyl succinate	143	(154)[a]
Ethyl acetate	122	(141)
Ethyl benzoate	122	(104)
Diethyl oxalate	122	(200)
Ethyl β-hydroxy-butyrate	122, 150	(104)
Dibutyl tartrate	100	(151)
Amyl acetate	88	(141)

[a] Values in parentheses are relative mobilities of acid anions corresponding to the respective ester acyl groups.

contain a high concentration of ethyl xanthate along with potassium oxalate.

Alkaline hydrolysis of esters is known to proceed extremely rapidly in aqueous dimethyl sulphoxide[34,35], and brief pre-treatment of esters with potassium hydroxide in dimethyl sulphoxide followed by the addition of carbon disulphide resulted in the formation of xanthates in the required amounts. As indicated in Table II, each ester yielded the expected alkyl xanthate. Ethyl β-hydroxybutyrate gave both ethyl and 2-carboxy-1-methylethyl xanthates, the latter being derived from the free β-hydroxy acid anion.

The relative mobilities of the acid anions corresponding to the acyl groups of the esters are also listed in Table II. When required, acid anions and alkyl xanthates were located simultaneously on pherograms by selecting spray reagents reactive to both species. The method thus affords simultaneous identification of the component alcohols and acids of esters and offers an alternative to standard procedures[36] which involve the formation, in separate tests, of appropriate crystalline derivatives.

The conditions of hydrolysis of the esters and xanthation of the carbohydrate derivatives were similar in that both were conducted in dimethyl sulphoxide containing potassium hydroxide. Valid results were therefore not expected from experiments performed to detect the presence of free hydroxyl groups in carbohydrate esters and it was found that some hydrolysis of carbohydrate benzoates, acetates and carbonates occurred during xanthation in dimethyl sulphoxide. Compounds 27–31 and 36 contain glycosidic, ether, cyclic acetal and cyclic ketal bonds and each formed the expected mono-xanthate only. Evidently these bonds are stable under the prescribed conditions and valid conclusions may be drawn from xanthate tests performed on compounds containing them.

Inorganic ions

Carbonate and some sulphur-containing anions are always present in aqueous solutions of xanthates[10]. A summary of results of the electrophoresis of these and related anions in $NaHCO_3$ solution is given in Table III.

Trithiocarbonate (CS_3^{2-}) and sulphide form as byproducts during xanthation of alcohols by reaction of carbon disulphide with the alkali metal hydroxide[37,38]. Alkaline solutions of xanthates allowed to stand unprotected from air slowly accumulate thiosulphate, which arises, at least in part, by direct oxidation of sulphide. The

TABLE III

RELATIVE RATES OF MIGRATION OF SULPHUR-CONTAINING INORGANIC ANIONS
The standard conditions of electrophoresis given in Table I were used.

Anion	$M_N \times 100$
Trithiocarbonate	257
Thiosulphate	249
Sulphide	249
Sulphate	209
Trithiopercarbonate	207
Tetrathionate	206
Sulphite	185

presence of these ions in dilute xanthate preparations was demonstrated after their rapid electrophoretic separation from the organic derivatives. Sulphide and thiosulphate have identical mobilities, not only in $NaHCO_3$ solution but also in sodium carbonate, sodium hydroxide and acetate buffer at pH 6 (compare GROSS[39]). Owing to its volatility, the detection of sulphide on pherograms at the lower values of pH was accomplished only by the immediate application of nitroprusside to the papers while they were still wet with the electrolyte. When dried, the same papers were oversprayed with chromium trioxide–permanganate–sulphuric acid for the detection of thiosulphate. The identity of thiosulphate was established on other pherograms which were dried in the ordinary way and subjected to the specific test for thiosulphate described in the Experimental section. The test was performed without delay because other sulphur-containing ions, including the xanthates, slowly formed thiosulphate on the papers in the presence of atmospheric oxygen and moisture.

INGRAM AND TOMS[18] noted, by means of ultraviolet absorption spectroscopy, that trithiocarbonate in aqueous solution in contact with air was converted to another ion which they considered to be trithiopercarbonate (COS_3^{2-}). We have followed this conversion by paper electrophoresis under the standard conditions. Fresh solutions of trithiocarbonate yielded a single, yellow, comet-shaped spot, the "tail" being formed, probably, by partial conversion to the other anion during electrophoresis. Solutions exposed to air for several hours prior to electrophoresis were resolved into two yellow spots, the faster-moving of which was due to trithiocarbonate. The spots were always connected by a faint yellow streak. The anion responsible for the slower-moving spot became preponderant in older solutions of trithiocarbonate and was probably identical with that described by INGRAM AND TOMS as trithiopercarbonate. It has been designated as such in Table III. Further exposure of the solutions to air resulted in the gradual disappearance of the latter ion with simultaneous increase in the concentration of thiosulphate. After three days, thiosulphate was the only anion detected.

Trithiocarbonate and trithiopercarbonate were shown to be present in solutions of xanthates along with sulphide and thiosulphate. The four ions occurred in proportions which varied according to method of preparation, concentration and age of the samples and the changing pattern of spots observed with aging solutions was consistent with the foregoing discussion of the reactivities of the ions. The transformation of trithiocarbonate to trithiopercarbonate in highly alkaline xanthate solutions was slower than that noted in aqueous solutions of the pure salt. This is in agreement with

the work of INGRAM AND TOMS, who showed that the rate of conversion varied inversely with the concentration of sodium hydroxide.

The electrophoresis of trithiocarbonate and trithiopercarbonate does not appear to have been described previously. The order of separation of the other anions given in Table III corresponds with that recorded by other workers using different electrolytes[39-43].

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CHROM. 3628

THE PAPER ELECTROPHORETIC STUDY OF ION PAIR FORMATION

III. ION PAIR FORMATION WITH QUATERNARY AMMONIUM IONS

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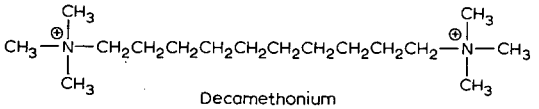
SUMMARY

The ion pair formation between quaternary ammonium ions and various anions was studied by high voltage paper electrophoresis.

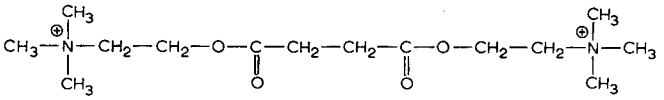
There seems to be little ion pair formation in 0.1 *N* solutions but quite considerable interaction in 1 *N* solutions.

INTRODUCTION

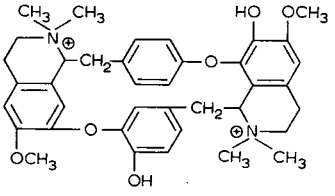
In the two previous papers of this series we have shown that ion pair formation can be used to improve the separation of metal complexes by paper electrophoresis.



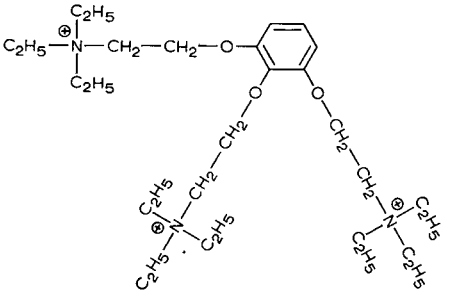
Decamethonium



Succinylcholine



d-Tubocurarine



Sincurarine

Fig. 1. Structural formulae of the quaternary ammonium compounds studied.

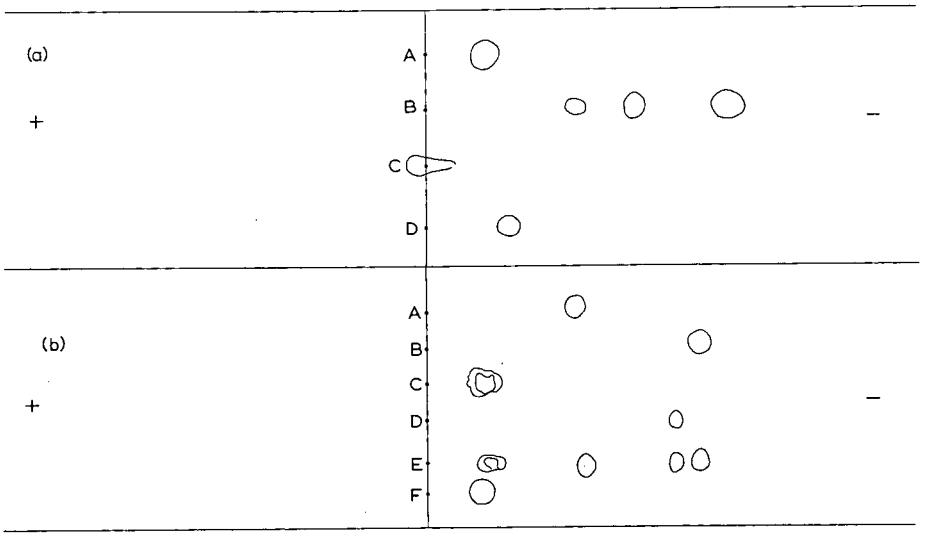


Fig. 2. Separation of some curarising compounds ($^1/_3 \times$ actual size). (a) Electrolyte: sodium trichloroacetate 1 *N* at pH ~ 4.5. 1500 V for 1 h on Whatman No. 1 paper. A = Sincurarine; B = succinylcholine (centre spot is succinylcholine, the other two spots are hydrolysis products); C = *d*-tubocurarine; D = H_2O_2 (for measurement of electroosmotic flow). (b) Electrolyte: sodium dichloroacetate 1 *N* at pH 4.5. 1500 V for 1 h. A = Sincurarine; B = succinylcholine (solid, freshly dissolved in water, and does not show spots of hydrolysis products); C = *d*-tubocurarine; D = decamethonium; E = mixture of A–D; F = H_2O_2.

Quaternary ammonium salts are a group of compounds which usually yield multispots in paper chromatography due to ion pair formation[1] and we thus decided to continue our studies by examining several quaternary ammonium compounds, especially those of pharmaceutical interest.

EXPERIMENTAL AND RESULTS

High-voltage paper electrophoresis with a Camag apparatus was employed as described before[2] (Whatman No. 1 paper at *ca.* 15°C with 1500 V for 30 min and H_2O_2 run for the measurement of electro-osmotic flow). All compounds were detected with Dragendorff's reagent.

Table I shows the distances moved by $Co(NH_3)_6^{3+}$ (so as to have some comparison with metal complexes studied previously) tetramethylammonium ion, tetraethylammonium ion and sincurarine (Fig. 1) a trivalent cation. The last two columns show the movement of tetraethylammonium relative to tetramethylammonium, and sincurarine relative to tetramethylammonium.

Table II shows the movement of several quaternary ammonium compounds used in pharmacy for their curare-like action.

Succinylcholine usually yields two to three spots owing to rapid hydrolysis in solution (see Fig. 2). Only choline and succinylmonocholine were detected here. In Table II the distance moved by the original compound is indicated.

Fig. 3 shows the movement of the four compounds of Table II in a graphical form. The different separation effects which can be obtained in various anions become evident.

TABLE I

ELECTROPHORETIC MOVEMENT OF QUATERNARY AMMONIUM IONS

Electrolyte	*Concentration (N)*	*Distance moved in mm in 30 min with 1500 V*				*Ratio of movement of* NEt_4^+/NMe_4^+	*Ratio of movement of sincurarine/* NMe_4^+
		$Co(NH_3)_6^{3+}$	*Sincurarine*	NMe_4^+	NEt_4^+		
LiCl	0.1		57	86	59	0.69	0.66
LiCl	0.5		50	91	62	0.68	0.55
LiCl*	1.0		26	51	34	0.67	0.51
NaBr	0.1	110	49	87	56	0.65	0.56
NaBr	0.5	127	41	93	57	0.62	0.44
NaBr**	1.0	50	12	40	23	0.58	0.30
$NaClO_4$	0.1	101	27	76	52	0.69	0.35
$NaClO_4$	0.5	104	18	72	43	0.60	0.25
$NaClO_4$*	1.0		0	34	18	0.52	0.00
Li_2SO_4	0.1	21	51	77	58	0.75	0.66
Li_2SO_4	0.5	11	58	82	64	0.78	0.71
Li_2SO_4	1.0	13	61	84	66	0.79	0.73
AcH–AcNa	0.1	134	73	105	70	0.67	0.69
AcH–AcNa (pH $\simeq$ 4.5)	0.5	94	44	70	49	0.70	0.63
AcH–AcNa	1.0	94	48	84	53	0.63	0.57
$CH_2ClCOOH$	0.1	105	53	82	55	0.67	0.65
$CH_2ClCOOH$ (pH $\simeq$ 4.5)	0.5	103	48	86	57	0.66	0.56
$CH_2ClCOOH$	1.0	97	43	91	55	0.61	0.47
$CHCl_2COOH$	0.1	101	45	78	55	0.70	0.58
$CHCl_2COOH$ (pH $\simeq$ 4.5)	0.5	108	33	84	50	0.60	0.39
$CHCl_2COOH$	1.0	64	14	52	27	0.52	0.27
CCl_3COOH	0.1	111	41	83	55	0.66	0.49
CCl_3COOH (pH $\simeq$ 4.5)	0.5	94	16	74	39	0.53	0.22
CCl_3COOH	1.0	65	− 8	51	16	0.31	

* With 1000 V for 30 min.
** With 1000 V for 25 min.

DISCUSSION

Several interesting points emerged from the results in Tables I and II. The ratio of the movement of tetraethylammonium to tetramethylammonium is more or less constant for 0.1 *N* electrolyte concentrations. This would indicate that there is similar or no ion pair formation at this concentration. The ratio increases considerably at 1 *N* electrolyte concentration for some anions, *e.g.*, for perchlorate, dichloroacetate and trichloroacetate.

The ratio of the movement of sincurarine to tetramethylammonium ion is around 0.66 (actually 0.66, 0.66, 0.69 and 0.65) for 0.1 *N* chloride, sulphate, acetate, monochloroacetate and below that for bromide, perchlorate, dichloroacetate and trichloroacetate indicating that some ion pair formation seems to take place even in 0.1 *N* solutions. Zero movement is obtained in 1 *N* perchlorate and anionic movement in 1 *N* trichloroacetate and these are also the anions which showed most interaction with cobalt complexes such as the tris-*o*-phenanthroline $Co(III)^{3+}$.

Somewhat unexpected results were obtained with sulphate. Here the difference in movement decreases with the increase in the concentration of the anion. As the distance moved by the tetramethylammonium ion is of the same order as in the other electrolytes this does not seem to be caused by unduly strong ion pair formation by this ion. We have no explanation for this phenomenon.

TABLE II

ELECTROPHORETIC MOVEMENT OF CURARE AGENTS

Electrolyte	*Concentration*	*Distances moved in mm in 30 min with 1500 V*			
	(*N*)	*Deca-methonium*	*Succinyl-choline*	*Sin-curarine*	*d-Tubo-curarine*
LiCl	1		29 (34*)	17	7
$NaClO_4$	1		6 (24*)	− 4	− 4 T
Li_2SO_4	1	62	60	51	17
AcH–AcNa (pH ≈ 4.5)	1	54	58	42	14
$CH_2ClCOOH$ (pH ≈ 4.5)	1	54	58	36	11
$CHCl_2COOH$ (pH ≈ 4.5)	1	37	42	20	3
CCl_3COOH (pH ≈ 4.5)	1	9	24	− 5	−15

* Hydrolysis product in brackets.
T = comet.

Table II and Fig. 3 illustrate the main aim of the investigation, *i.e.*, that ion pair formation can be profitably employed to change and improve separations by paper electrophoresis.

Incidentally, ion pair formation need not increase with the number of charges in the molecule. *d*-Tubocurarine has two quaternary ammonium groups and is more anionic than sincurarine with three. The polarity and size, etc., of the molecule are equally important.

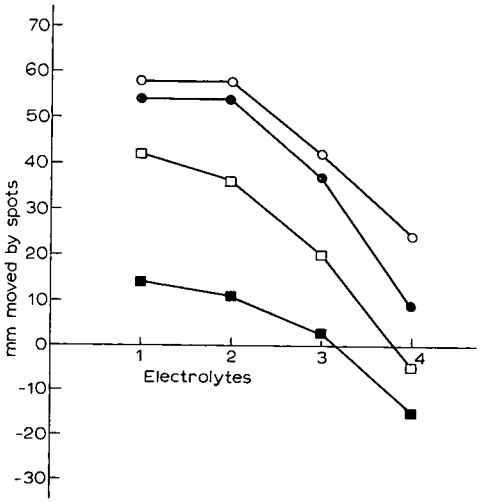


Fig. 3. Graphical representation of the movement of curare-like compounds in 1 N acetate and mono-, di- and trichloroacetate. ● = Decamethonium; ○ = succinylcholine; □ = sincurarine; ■ = *d*-tubocurarine. 1 = CH_3COOH–CH_3COONa 1 N; 2 = $CH_2ClCOOH$ 1 N; 3 = $CHCl_2COOH$ 1 N; 4 = CCl_3COOH 1 N; 2, 3 and 4 adjusted to pH $\simeq$ 4.5 with NaOH.

Most theoretical treatments of paper electrophoresis (for example ref. 3) ignore ion pair formation, and perhaps justly so, because it plays only a negligible role in 0.1 N or less concentrated electrolytes. In an apparatus with adequate cooling (*e.g.* the Camag apparatus) there is no reason why 1 N electrolytes should not be used and at this concentration not only the size, shape, charge and degree of ionisation of an ion decides its movement but, as shown here, interactions with the surrounding anions play an essential role. Quaternary ammonium compounds were chosen for this study because they are strong bases and hence their movement is not influenced by the pH of the electrolyte and because their separation by chromatographic methods is less satisfactory than for many other classes of compounds. In principle ion pair formation should also be useful in altering separations of other classes of compounds which can be made to migrate in an electric field.

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CHROM. 3600

RÉSOLUTIONS CHROMATOGRAPHIQUES DE RACÉMIQUES SUR ÉCHANGEURS D'IONS NATURELS, OPTIQUEMENT ACTIFS

I. RÉSOLUTION DE BASES ORGANIQUES SUR L'ACIDE POLYGALACTURONIQUE

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SUMMARY

Chromatographic resolution of racemates on natural optically active ion-exchangers. I. Resolution of racemic bases on polygalacturonic acid

It has been shown experimentally that chromatographic resolution of racemic bases on polygalacturonic acid is possible according to any one of the following equations:

$$(1)\quad \begin{cases} R_sNH_2 + HOOCR_g \rightleftharpoons R_sNH_3\cdot OOCR_g \\ R_rNH_2 + HOOCR_g \rightleftharpoons R_rNH_3\cdot OOCR_g \end{cases}$$

$$(2)\quad \begin{cases} R_sNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_sNH_2 + NH_4OOCR_g \\ R_rNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_rNH_2 + NH_4OOCR_g \end{cases}$$

$$(3)\quad \begin{cases} R_sNH_3Cl + NaOOCR_g \rightleftharpoons R_sNH_3OOCR_g + NaCl \\ R_rNH_3Cl + NaOOCR_g \rightleftharpoons R_rNH_3OOCR_g + NaCl \end{cases}$$

It was found that the degree of swelling of the polygalacturonic acid in the system water–methanol–ether strongly affects the effectivity of the resolution. The latter increases as the degree of swelling increases, reaching its highest value at an optimal swelling of 20–25 ml/g.

INTRODUCTION

Un grand nombre de publications traitent de l'utilisation d'adsorbants naturels, optiquement actifs, pour la séparation chromatographique de racémiques[1–3]. En ce sens, l'amidon[4,5], la cellulose[6–9], certaines substances protéiques, et d'autres composés optiquement actifs, le quartz, etc., ont été utilisés avec succès.

En outre, différents échangeurs d'ions optiquement actifs[10,11] ont été synthétisés et utilisés au cours des dix dernières années, pour séparer la lysine, l'acide

mandélique, et d'autres racémiques — cependant — jusqu'à présent les polyélectrolytes naturels, telles les substances pectiques (et plus spécialement l'acide polygalacturonique) et l'algine, n'ont pas attiré l'attention des rechercheurs. Mais les susdits produits étant accessibles et d'homogénéité chimique relativement grande, nous les avons jugés très utiles pour séparer chromatographiquement des bases racémiques.

Jusqu'ici un seul essai[12] a été publié sur ces études, leur auteur ayant utilisé de la chitine déacéthylisée en guise d'anionite optiquement active.

Dans une communication préliminaire[13], nous avons posé les conditions qui favoriseraient l'usage d'échangeurs d'ions optiquement actifs pour le dédoublement chromatographique de bases ou d'acides racémiques.

RAPPEL THÉORIQUE

En nous basant sur des considérations théoriques d'ordre général nous avons pu admettre que la résolution chromatographique d'une base racémique ($R_sNH_2 + R_rNH_2$) pourrait être réalisée sur les cellules élémentaires (R_gCOOH) de l'acide polygalacturonique, selon les réactions suivantes:

$$(1)\ \begin{cases} R_sNH_2 + HOOCR_g \rightleftharpoons R_sNH_3\cdot OOCR_g \\ R_rNH_2 + HOOCR_g \rightleftharpoons R_rNH_3\cdot OOCR_g \end{cases}$$

$$(2)\ \begin{cases} R_sNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_sNH_2 + NH_4OOCR_g \\ R_rNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_rNH_2 + NH_4OOCR_g \end{cases}$$

$$(3)\ \begin{cases} R_sNH_3Cl + NaOOCR_g \rightleftharpoons R_sNH_3OOCR_g + NaCl \\ R_rNH_3Cl + NaOOCR_g \rightleftharpoons R_rNH_3OOCR_g + NaCl \end{cases}$$

En principe, il est possible d'établir des conditions assurant différents comportements des deux antipodes du mélange racémique pour chacune des réactions.

Nous avons expérimentalement confirmé cette hypothèse.

PARTIE EXPÉRIMENTALE

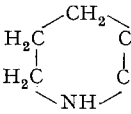
Matériel

Nous avons effectué des chromatogrammes sur colonne, utilisant l'acide polygalacturonique, obtenu de moelles d'Helianthus à 96 % de pureté[14], dont le degré de gonflement dans le système eau–méthanol–éther dépend des rapports de ces trois composants au cours du traitement préalable de l'acide polygalacturonique et se traduit par le volume (en ml) d'un gramme d'acide polygalacturique.

Bases utilisées

(±)-*thréo* $C_6H_5\cdot CH(NH_2)\cdot CH(C_6H_5)\cdot COOCH_3$ (I)

(±)-*érythro* $C_6H_5\cdot CH(NH_2)\cdot CH(C_6H_5)\cdot COOCH_3$ (II)

(±) — thréo  (ritaline) (III)

Méthodes

Essais I–VII (*Tableaux II et III*). Dans la colonne remplie d'acide polygalacturonique au juste degré de gonflement nous avons introduit la base sous forme de solution diluée dans du méthanol–ether (1:1). Nous avons recueilli de deux à sept fractions d'éluats; ensuite nous avons fractionné la colonne elle-même en deux à trois fractions que nous avons extraites avec de l'acide chlorhydrique (1 % dans du méthanol). Nous avons ensuite neutralisé avec du bicarbonate de sodium solide et fait évaporer le solvant. À fin d'éliminer les traces d'acides polygalacturoniques éventuellement entraînées nous avons traité les résidus avec 20 ml de bicarbonate de sodium (5 %). La base a été alors extraite avec de l'éther. Une fois l'éther évaporé, les résidus ont été dissous à chaud dans de l'hexane (10 ml) et filtrés. Après la filtration l'hexane a été évaporé. Les substances basiques sèches obtenues ont été dissoutes dans 5–10 ml de chloroforme et leur activité optique a été mesurée avec un polarimètre "Jouan". Les conditions expérimentales de chacune des chromatographies réalisées sont reportées sur le Tableau I.

TABLEAU I

CONDITIONS EXPÉRIMENTALES

		Numéro de l'essai						
		I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Hauteur du remplissage de la colonne (mm)		125	125	120	120	115	110	125
Diamètre de la colonne (mm)		14	11	11	14	18	15	19
Poids de l'acide polygalacturonique dans la colonne (g)		5.2	2.3	1.1	0.85	1.1	0.85	2.2
Degré de gonflement de l'acide polygalacturonique (ml/g)		3.5	5	10	23	27	23	14
Base racémique ajoutée (g) au mélange de méthanol–éther, 1:1 (ml)		0.5/100	0.5/100	0.5/100	0.5/100	0.5/100	0.5/100	0.5/100
Vitesse de l'élution (ml/h)		35	35	35	35	35	100	170
Volume de différentes fractions éluats (ml)	I	50	100	100	100	100	100	200
	II	50	420	100	100	90	100	600
	III	50	—	100	80	50	100	350
	IV	50	—	80	20	50	100	—
	V	125	—	90	70	100	100	—
	VI	—	—	—	—	—	100	—
	VII	—	—	—	—	—	40	—

Essai VIII (*Tableau IV*). Nous avons dissous dans 5 ml d'un mélange de méthanol (85 %)–éther (1:1) de la base I, mélangée à 0.5 g d'acide polygalacturonique en poudre. Vingt-quatre heures après le sel obtenu a été introduit dans la colonne chromatographique (diamètre 18 mm) préalablement remplie de 4 g d'acide polygalacturonique au degré de gonflement de 17 ml/g.

Une solution de méthanol–éther (1:1) a été utilisée comme solvant. La colonne était de 290 mm. Nous avons élué avec de l'ammoniaque (0.1 %) dilué dans du méthanol–éther (1:1) [45 ml de méthanol (85 %), 50 ml d'éther, 5 ml d'eau ammoniacale (2 %)]. Vitesse de l'élution: 170 ml/heure. Nous avons recueilli 30 fractions

TABLEAU II

EFFETS DU DEGRÉ DE GONFLEMENT SUR L'EFFICACITÉ DE LA SÉPARATION

I *Degré de gonflement = 3.5 ml/g*			*II* *Degré de gonflement = 5 ml/g*			*III* *Degré de gonflement = 12 ml/g*		
No. de la fraction	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$	*No. de la fraction*	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$	*No. de la fraction*	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$
1	0.017	0	1	0.004	0	1	0.005	+3.2
2	0.146	+0.2	2–6	0.011	+6.3	2	0.020	+6.4
3	0.045	+0.2	7[a]	0.019	+7.3	3	0.023	+5.1
4	0.005	<+0.1	8[a]	0.204	+0.1	4	0.035	+5.8
5	0.004	<+0.1	9[a]	0.147	−0.5	5	0.036	+2.6
6[a]	0.102	−0.1				6[a]	0.123	−1.4
7[a]	0.118	−0.2				7[a]	0.087	−1.3
						8[a]	0.092	−1.9

[a] Substance (base) extraite de la colonne après achèvement de la chromatographie.

de 25 ml, dont, après neutralisation jusqu'à pH 7 avec de l'acide acétique (5 %), le solvant est évaporé. Les résidus ont été traités comme dans essais I–VII.

Essai IX (*Tableau V*). Nous avons fait couler 0.5 g de chlorhydrate de la base II, dilué dans 50 ml de méthanol, à travers une colonne de 26 cm de diamètre, remplie, jusqu'à la hauteur de 100 mm de polygalacturonate de sodium de degré de gonflement de 14 ml/g.

Le solvant utilisé a été le méthanol. L'élution a été réalisée, elle aussi, avec du méthanol, à la vitesse de 35 ml/h. Nous avons recueilli trois fractions de 30 ml et une quatrième fraction de 40 ml. La masse de la colonne a été divisée en deux parties, et nous avons procédé à l'extraction de chacune d'elles avec de l'acide chlorhydrique (2 % de méthanol). Les extraits et les éluats ont été ensuite neutralisés avec du bicarbonate de sodium solide. Une fois le solvant évaporé, les résidus ont été traités comme dans essais I–VII.

RÉSULTATS ET CONCLUSIONS

Nous avons étudié les effets du degré de gonflement de l'acide polygalacturonique dans des conditions assurant la résolution selon la réaction 1. Les résultats obtenus sont présentés dans le Tableau II. Nous voyons qu'il existe un degré de gonflement optimal de l'acide polygalacturonique, qui pour le système éther–méthanol–eau est de 20–25 ml/g environ. C'est dans ces conditions que nous avons procédé à la chromatographie des autres bases racémiques. Les résultats obtenus (Tableau III) prouvent qu'il est possible de résoudre des bases racémiques avec de l'acide polygalacturonique selon la réaction 1.

Pour étudier la deuxième réaction nous avons effectué la chromatographie du sel de la base II avec l'acide polygalacturonique. La colonne utilisée a été remplie d'acide polygalacturonique (degré de gonflement = 17 ml/g); l'éluant utilisé a été l'ammoniaque 0.1 % dans du méthanol–éther. Les résultats polarimétriques figurent dans le Tableau IV. Il en résulte que dans les conditions (de la réaction 2) la séparation

IV *Degré de gonflement = 23 ml/g*			*V* *Degré de gonflement = 27 ml/g*		
No. de la fraction	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$	*No. de la fraction*	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$
1	0.005	+ 3.1	1	0.002	+1
2	0.083	+20.1	2	0.027	+5.8
3	0.047	+ 3.7	3	0.024	+5
4	0.013	+ 0.9	4	0.024	+4.5
5	0.021	− 0.8	5	0.031	+2.7
6[a]	0.106	− 5.6	6[a]	0.120	0
7[a]	0.089	− 6.6	7[a]	0.081	−1.2
8[a]	0.044	− 8.3	8[a]	0.089	−2.4

chromatographique des bases racémiques est possible. Il est intéressant de signaler que le pH des éluats contenant l'antipode (+) est de 6.5 à 7, mais si le pH atteint 7.5 on est en présence d'une inversion. Ce fait permet d'admettre qu'une résolution plus efficace des deux antipodes serait possible dans la réaction 2, si le pH de l'éluant était strictement régularisé.

Nous avons finalement étudié les possibilités de résolution des chlorhydrates d'une base chimique donnée sur du polygalacturonate de sodium (réaction 3). Nous avons chromatographié le chlorhydrate de la base I. Les résultats obtenus (Tableau V) prouvent que, dans les conditions établies, une pareille résolution est possible bien qu'avec une efficacité moindre.

TABLEAU III

ESSAIS DE SÉPARATION DES BASES RACÉMIQUES I, II ET III D'APRÈS LA RÉACTION I

VI (±)-*thréo ester* (*I*)			*IV* (±)-*érythro ester* (*II*)			*VII* (±)-*Ritaline*		
No. de la fraction	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$	*No. de la fraction*	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$	*No. de la fraction*	*Poids de la base dans la fraction (g)*	$[\alpha]_{20}^{D}$
1	0.007	+5.7	1	0.005	+ 3.1			
2	0.090	+6.7	2	0.083	+20.1	1–2	0.025	+3.4
3	0.060	+1.8	3	0.047	+ 3.7	3–10	0.092	+4.2
4	0.041	+1.7	4	0.013	+ 0.9	11–17	0.060	+4.4
5	0.030	+2.2	5	0.021	− 0.8	18[a]	0.003	0
6	0.022	+1.4	6[a]	0.106	− 5.6	19[a]	0.002	0
7	0.012	+1.9	7[a]	0.089	− 6.6	20[a]	0.235	−1.8
8[a]	0.112	−2.1	8[a]	0.044	− 8.3			
9[a]	0.077	−2.8						

[a] Substance (base) extraite de la colonne après achèvement de la chromatographie.

TABLEAU IV

ESSAI VIII–SÉPARATION DE LA BASE I D'APRÈS LA RÉACTION 2

No. de la fraction	*pH de l'éluat*	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$
1–12	<6.5	<0.001	0
13	6.5	0.012	+10
14	6.5	0.019	+ 6.5
15	7	0.042	+ 4
16–17	7.5	0.103	+ 2
18	7.5	0.044	+ 2
19	7.5	0.051	− 1.5
20	7.5	0.043	− 3
21	7.5	0.026	− 4.5
22	7.5	0.026	−23
23	7.5	0.017	−18
24–30	7.5	<0.001	0

TABLEAU V

ESSAI IX–SÉPARATION DU CHLORHYDRATE DE LA BASE II SUR DE POLYGALACTURONATE DE SODIUM

No. de la fraction	*Poids de la base dans la fraction (g)*	$[\alpha]_D^{20}$
1–2	0.001	0
3	0.068	+3
4	0.120	+1.7
5[a]	0.038	−1.3
6[a]	0.080	−2.2

[a] Substance (base) extraite de la colonne après achèvement de la chromatographie.

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RÉSUMÉ

Les auteurs montrent que la résolution chromatographique des bases racémiques sur l'acide polygalacturonique est réalisable selon une des réactions suivantes:

$$(1)\begin{cases} R_sNH_2 + HOOCR_g \rightleftharpoons R_sNH_3\cdot OOCR_g \\ R_rNH_2 + HOOCR_g \rightleftharpoons R_rNH_3\cdot OOCR_g \end{cases}$$

$$(2)\begin{cases} R_sNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_sNH_2 + NH_4OOCR_g \\ R_rNH_3\cdot OOCR_g + NH_3 \rightleftharpoons R_rNH_2 + NH_4OOCR_g \end{cases}$$

$$(3)\begin{cases} R_sNH_3Cl + NaOOCR_g \rightleftharpoons R_sNH_3OOCR_g + NaCl \\ R_rNH_3Cl + NaOOCR_g \rightleftharpoons R_rNH_3OOCR_g + NaCl \end{cases}$$

Ils signalent, en outre, que le degré de gonflement de l'acide polygalacturonique dans le système eau–méthanol–éther influence fortement l'efficacité de la résolution. Ils obtiennent de meilleurs résultats en utilisant de l'acide polygalacturonique de degré de gonflement de 20–25 ml/g environ.

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Notes

CHROM. 3676

Three-dimensional model for the optimum column conditions in gas chromatography

As is well known, the concept of the height equivalent to a theoretical plate, HETP, has been introduced and extensively used in gas chromatography. KLINKENBERG AND SJENITZER[1] treated the HETP concept mathematically on the basis of Poisson distribution. VAN DEEMTER *et al.*[2] developed the famous kinetic theory. In addition, a new theory[3,4], in which the pressure drop along the column was taken into consideration, was formulated. This theory[4] predicts that HETP is related to the outlet carrier gas flowrate, u_o, by an equation of the following form:

$$H = \frac{B}{u_o} + C\sqrt{u_o}$$

This relationship was confirmed experimentally[5].

On the other hand, HETP varies remarkably with column temperature[6]. In the present report, the effect of column temperature and of carrier gas flowrate on HETP was studied and the relationship was found to be expressed suitably by a three-dimensional model.

Experimental

A Hitachi gas chromatograph, type KGL-2A, equipped with a thermal conductivity detector, was used. The column, 2 m in length and 0.4 cm in diameter, was a copper tube packed with a stationary phase of 10 % Apiezon grease L on 60–80 mesh Diasolid M. Helium gas flowrate was measured with a soap film meter and corrected for the saturated vapour pressure. The column inlet pressure was measured with a calibrated pressure gauge, and the outlet pressure was always kept at atmospheric pressure. The column temperature was maintained constant within *ca.* 0.1 %. The toluene sample was injected by means of a 10-μl syringe into the heated injection block which ensures rapid vaporization.

Results and discussion

The HETP varies with sample size. With decreasing sample size, from 5 μl to 0.6 μl, the plate number increased. The corresponding optimum column temperature also became higher with increasing sample size, as shown in Fig. 1. The plate number of the column was found to be almost independent of sample size, if one employs a sample smaller than 0.6 μl, as shown in Fig. 2. Thus the sample size was maintained at 0.4 μl throughout the experiment. Under such conditions the HETP can be considered to depend on only two variables, *i.e.*, column temperature and flowrate. Curve A in Fig. 3 shows the dependence of HETP on the absolute carrier gas flowrate

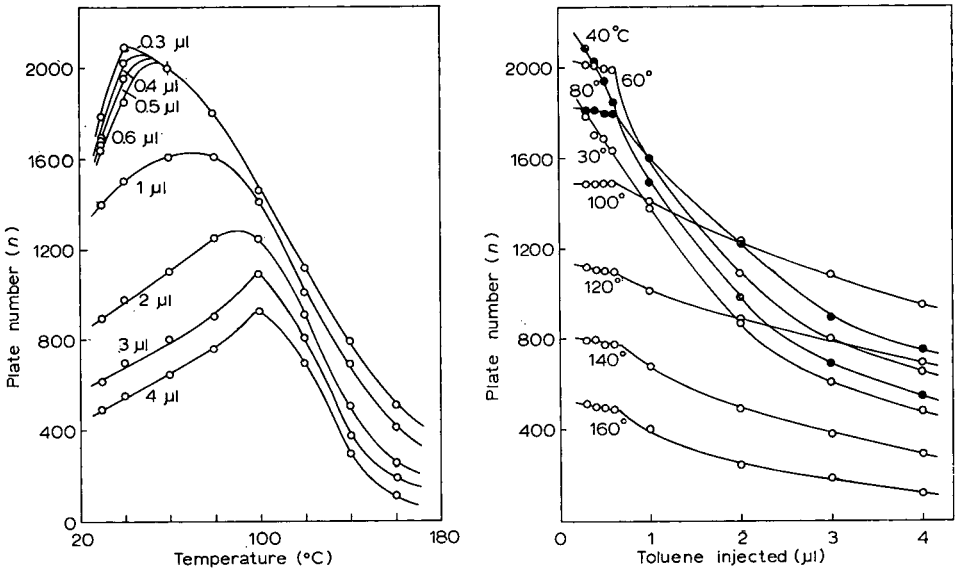

Fig. 1. Plot of plate number against column temperature. The figures show the sample amount injected. Sample: toluene. Column: 2 m long, 0.4 cm diam., 10% Apiezon grease L on 60–80 mesh Diasolid M.

Fig. 2. Variation of plate number with sample amount injected at various column temperatures.

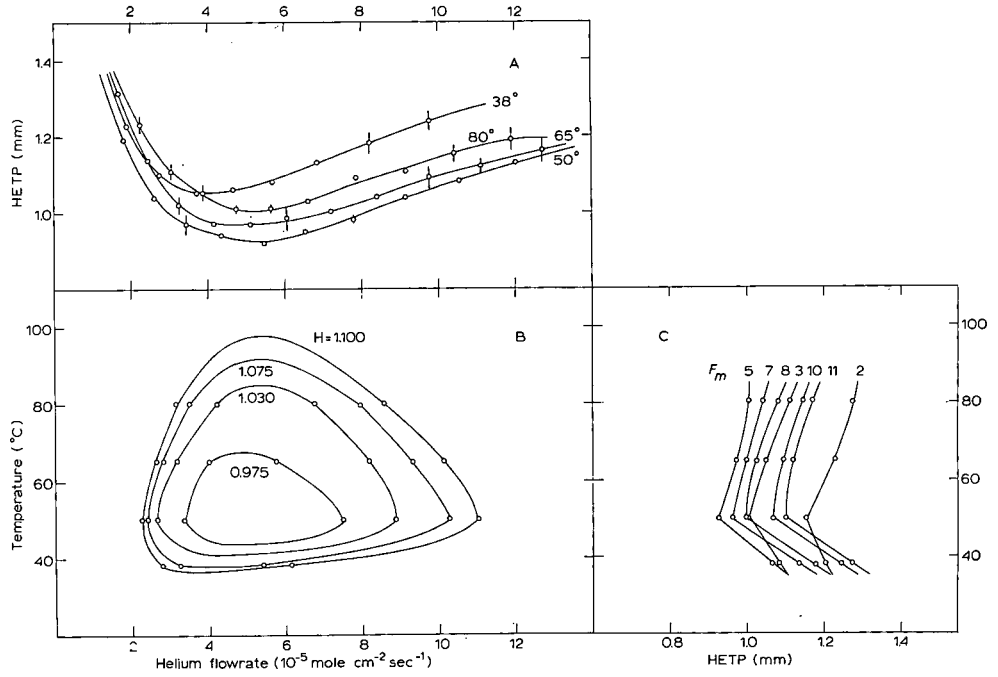

Fig. 3. The HETP plotted against absolute flowrate of the helium carrier gas at various column temperatures (curve A) and HETP plotted against column temperature at various absolute flowrates of carrier gas (curve C). Curve B shows the contour lines of HETP. The vertical lines in curve A denote the 95% confidence interval.

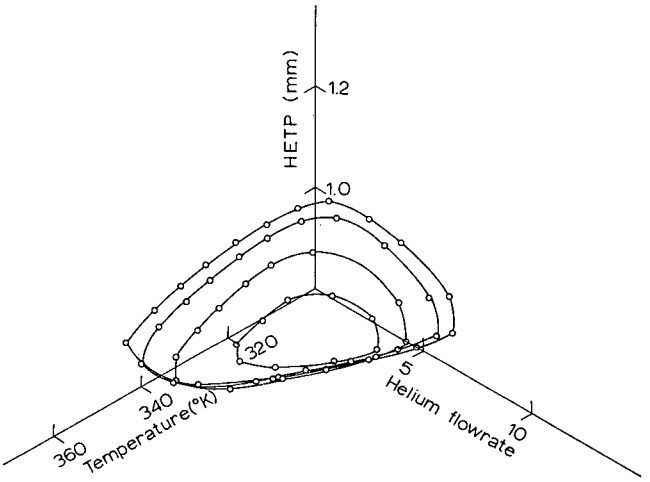

Fig. 4. Three dimensional model for the HETP expressed as the function of absolute carrier gas flowrate and column temperature (°K).

F_m at various temperatures. On increasing the column temperature from 50° to 80°, the H–F_m curve is shifted (lowered), and a minimum H-value was obtained at 50°, but at 38° the H–F_m curve is again shifted upwards and the column efficiency decreased. If we pick out the points that give the same value of HETP in curve A, Fig. 3 and plot them as a function of column temperature and flowrate, we can obtain the contour lines of HETP as shown in curve B, Fig. 3. The dependence of HETP on the column temperature is shown in curve C, Fig. 3. Illustrating these relations in oblique co-ordinates, we can obtain the conical diagram as shown in Fig. 4. With these diagrams one can obviously understand how the HETP depends on column temperature and the flowrate of carrier gas. The best condition of the column is given by the lowest point in Fig. 4.

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CHROM. 3711

Improved preparative collections from the Autoprep gas chromatograph

With the continual improvement of equipment, preparative-scale gas–liquid chromatography has become increasingly important for the isolation of many types of volatile compounds. However, the abrupt temperature change encountered at the column exit port frequently gives rise to stable aerosols which drastically reduce the efficiency of collection.

Application of the principle of the Cottrell precipitator for the collection of gas chromatograph eluates not otherwise trapped because of aerosol formation has been reported by several investigators[1–3]. The most recent paper[3] describes a modification primarily designed for the F & M model 770 preparative gas chromatograph.

We have devised a simple modification of the widely used Aerograph Model A-700 Autoprep (Varian Aerograph Inc., Walnut Creek, Calif.) (Fig. 1) to provide for the collection of aerosols by electrostatic precipitation. This instrument utilizes a

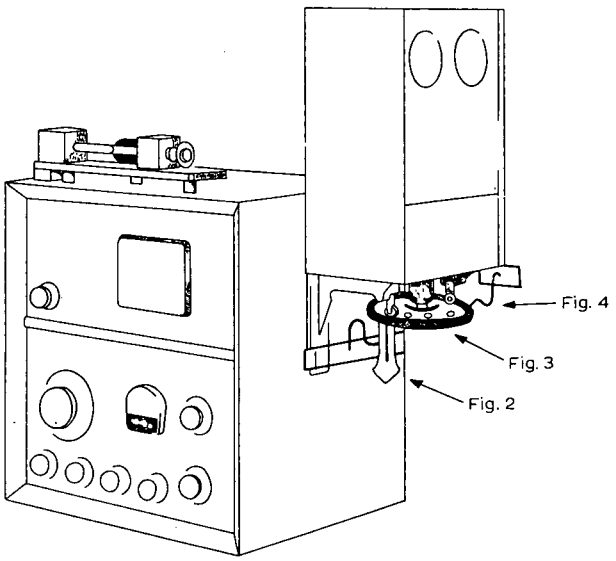


Fig. 1. Complete preparative chromatograph.

circular collector table to which collection bottles are attached. The collector table is rotated into place automatically upon activation by the recorder response so that the carrier gas and the vaporized sample are introduced serially into each collector bottle at the appropriate time.

Modification of the usual commercial collector bottle (Fig. 2a) allows the effluent from the chromatograph to pass through an electrostatic field which causes precipitation of the sample from the carrier gas. Cutting off the bent portion of the exit side (Fig. 2b) permits insertion of an inner electrode which is constructed of spring wire (A) embedded in a glass capillary (B) with epoxy resin and is held in the center

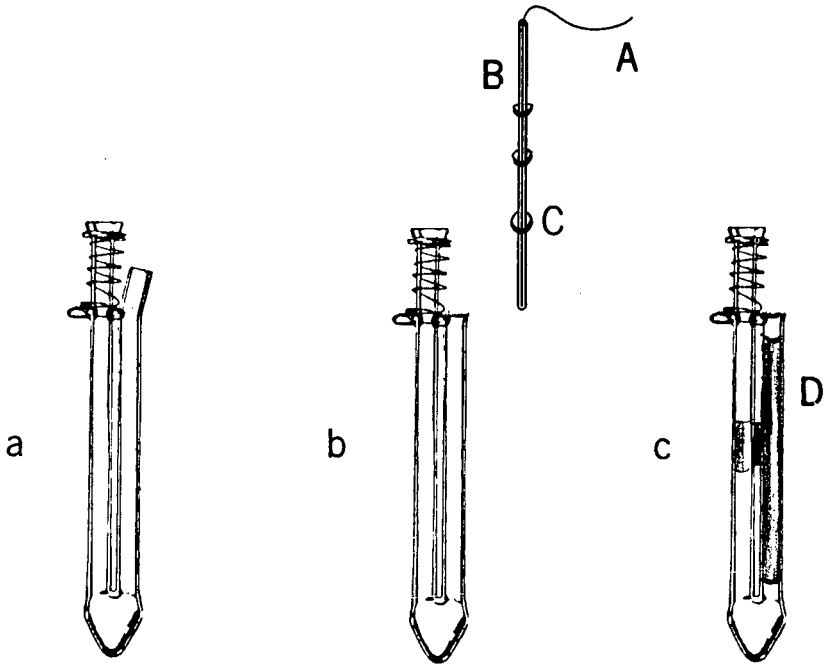


Fig. 2. Collector bottle detail (see text).

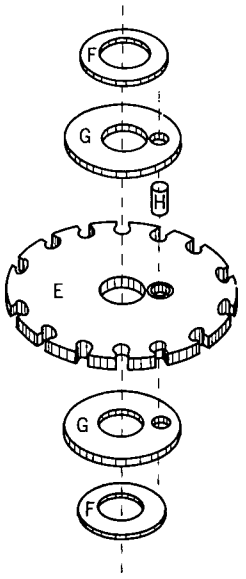
of the exit tube by Teflon spacers (C) cut from sheet with a cork-borer. This design permits withdrawal of the electrode to facilitate removal of the collected sample.

The outer electrode is formed by wrapping aluminum foil tape ("Permacel") (D) around the exit side of the collector bottle (Fig. 2c) and can be readily removed after the collection has been completed. Wide variation in the dimensions of collector bottles is possible so long as the effluent is passed through an electrostatic field of sufficient intensity.

In order to provide high voltage safely to the inner electrode A, the standard collector table E was modified as shown in Fig. 3. An upper conducting metal ring, F, was cemented with epoxy resin to a larger insulator ring G constructed of a 1/8 in. (3 mm) thickness of hardboard. A similar pair of rings was cemented to the lower side of the collector table, and the F rings were connected electrically by a Teflon-insulated piece of 12 gauge copper wire, H.

A sketch of the assembled collector (Fig. 4) shows the relationship of the modified units to each other. The upper end of each electrode wire and a spring wire brush, I, were arranged to maintain continuous contact with opposite F rings. To complete the high-voltage circuit, a short length of wire, J, was attached to the strip of insulation material K bolted to the collector mounting bracket. The wire was shaped to make contact with the metal foil on the collector bottle as it moved to the collection position. The metal chromatograph cabinet was grounded.

The high voltage required was supplied by a transformer with a 120 VAC primary and a 6000 VAC secondary (such as a commercial neon-light transformer). Voltage to the electrodes was varied by adjustment of the primary voltage with an autotransformer (Variac). To determine the voltage required for precipitation, a



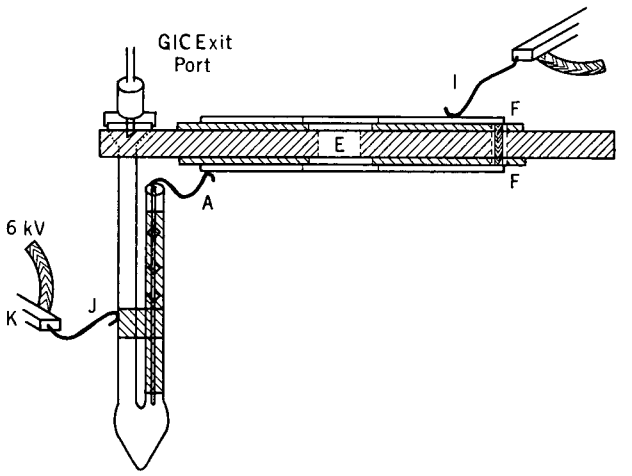


Fig. 3. Collector table detail (see text).

Fig. 4. Bottle in place for collection.

sample was injected with power at zero; when the desired fraction passed through the collector bottle, the voltage was increased until emergence of the aerosol from the bottle no longer was observed. A primary voltage of 50–60 VAC was required for the modified collector bottle described above.

This electrostatic collector has been used successfully with samples in which each fraction represented approximately 5 mg of collected material per injection on a 1.6 cm × 150 cm column; collection efficiency was essentially quantitative. Collected fractions were rechromatographed and checked by infrared spectroscopy for signs of degradation due to the electrostatic field; no evidence of degradation was ever observed.

A Plexiglass shield with an interlocking safety switch can be readily incorporated and provides complete safety for the operator.

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CHROM. 3712

A helium ionization detector of high sensitivity

It is widely accepted that the principle underlying the operation of the argon and helium ionization detectors is based upon the "Penning effect". Despite this there are a few instances where, using a helium ionization detector[1–3], the effect is incapable of accounting for the results. The impurity in each instance, neon, hydrogen, argon and nitrogen, gave either a wholly or partly negative response with a helium carrier gas of extreme purity.

In most of the work in which the "Penning effect" has been invoked to account for experimental results the detector pressure was either below about 50 torr[4–6] or above 700 torr[2,7]. It was, therefore, desirable to investigate the change in the detector response over a much wider range of pressures. A Berry detector was installed in an assembly and operated at pressures from about 5 torr to values in excess of 700 torr; this detector assembly is to be fully described elsewhere. The detector was connected on the upstream side, to a molecular sieve 5A column gas chromatograph via a flow restrictor and on the downstream side to a vacuum pump. The pressure was measured close to the detector outlet on the downstream side and the pressures recorded were not therefore the actual pressures within the detector; it is unlikely, however, that they will differ greatly from them. The gas chromatograph operating conditions were:

(i) carrier gas flow rate 70 ml/min at ambient pressure,
(ii) sample size 10 ml,
(iii) detector anode voltage set to give a current of 4.7×10^{-8} A with the carrier gas using a decade voltage supply, W. G. Pye & Co., Ltd., Cat. No. 12363,
(iv) radioactive source 100 mC tritium foil.

TABLE I

VARIATION OF DETECTOR CURRENT WITH ABSOLUTE PRESSURE

Absolute pressure (torr)	*Anode voltage (V)*	*Detector current* $\times 10^{-8}$ *A for impurity*				
		O_2	Ar	CH_4	N_2	H_2
5	297	58.5	32.3	14.9	8.6	5.3
10	284	90.2	67.3	31.9	16.7	10.4
14	268	91.8	87.0	46.3	23.9	14.1
16	255	74.2	82.4	45.9	25.1	14.5
20	243	35.1	55.0	32.8	17.7	11.6
26	228	9.2	28.9	19.0	10.6	7.5
33	218	0.8	11.0	8.4	4.3	3.1
40	218	— 1.8	4.9	4.3	2.2	1.2
53	224	— 2.4	1.2	2.1	0.6	0.4[a]
67	241	— 2.4	1.3[a]	1.9	0.3[a]	0.6[a]
86	265	— 2.0	1.3[a]	1.6	0.3[a]	0.9[a]
110	295	— 1.3	1.6[a]	1.8	0.4[a]	0.9[a]
213	394	1.9	2.3[a]	3.3	0.6[a]	1.2[a]
320	460	2.5	2.1[a]	3.5	0.5[a]	1.2[a]
757	680	2.4	1.6[a]	4.2	0.4[a]	1.0[a]

[a] These results are anomalous. The current quoted is the sum of the positive and negative parts of the peak.

Using a helium carrier gas of extreme purity[3] and a helium–impurity gas blend containing 12 v.p.m. of each of nitrogen, methane, argon and hydrogen and 23 v.p.m. of oxygen, a series of analyses was carried out at different pressures. In each case the standing current of the detector was maintained at a constant value by adjustment of the applied voltage when pure helium was flowing through the detector. The molecular sieve column was regenerated *in situ* at 400° for 72 h with a continuous purge of the helium carrier gas and was capable of separating oxygen and argon[8].

The results given in Table I show that: (1) anomalous responses are not obtained for any of the impurities tested when the pressure is less than about 40 torr, (2) a wholly negative response is obtained for oxygen in the pressure range 40–110 torr, and (3) in all cases a maximum is reached at about 14–16 torr, which is 10–60 times more sensitive than at atmospheric pressure.

It would appear therefore that if the "Penning effect" is able to account for the detector current obtained at 14–16 torr, and the rapid fall in current from 16 torr to about 40 torr is due to the loss of metastable atoms, then the current obtained at values in excess of this pressure must be associated with either the surviving metastable atoms or another process.

The increase in sensitivity obtained at low pressures is to be further investigated for a wider range of impurities and operating conditions, and a detector will be built based upon these findings.

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CHROM. 3630

Gas chromatography of isomeric fatty acid methyl esters

As an adjunct to a recent study[1] of the carboxylic acids present in Latakia tobacco leaf, separation of over twenty standard samples of methyl esters of isomeric saturated monocarboxylic acids containing 1–7 carbon atoms was achieved on various types of capillary column.

It would appear that no systematic study of the complete range of these lower isomeric compounds has been made on capillary columns. SCHOMBURG[2] has studied the retention characteristics of methyl esters of a large number of branched-chain acids of five or more carbon atoms on a wide range of stationary phases coated on capillary columns. VASIL'EV *et al.*[3] have reported the separation of the methyl esters of many lower isomeric acids, using a packed column of silicone grease.

Our aim in this paper is to show that separation of these closely related compounds can be achieved on capillary columns and that, by suitable choice of stationary phase, tentative identifications can be made on the basis of retention time.

The compounds investigated include the methyl esters of all saturated monocarboxylic acids containing 1–6 carbon atoms and as many isomers of 7 carbon atoms as were available at the time of the study.

Experimental

Preparation of compounds. Many of the compounds used were commercially available as methyl esters or as free carboxylic acids.

3-Methylpentanoic acid, 3-methylhexanoic acid, 4-methylhexanoic acid and 5-methylhexanoic acid were synthesised by a general method[4] using sodium diethylmalonate and the appropriate alkyl halide.

2-Methylbutyric acid, 2,2-dimethylbutyric acid and 2-methylhexanoic acid were prepared by a general method[5] employing the carboxylation of the corresponding Grignard reagent.

2-Ethylbutyric acid and 2,3-dimethylbutyric acid were prepared by an extension of a method of FISCHER *et al.*[6,7] using sodium ethyl cyanoacetate.

Conversion of acids to methyl esters was effected, in most cases, by refluxing with methanol–conc. sulphuric acid reagent. However, more sterically hindered acids, *e.g.* trimethylacetic acid, gave acceptable yields only when treated with methanol–boron trifluoride reagent.[8]

Gas chromatography. A Perkin-Elmer F.11 instrument, equipped with a flame-ionisation detector, was employed throughout.

The columns and conditions used for the separation were as follows:

(i) A stainless-steel open-tubular capillary column, 30 m × 0.25 mm I.D., coated with silicone-gum rubber SE-30 (SE30). Nitrogen carrier gas inlet pressure: 5 p.s.i.g. Operating temperature: linearly programmed from 35° at 4°/min.

(ii) A stainless-steel open-tubular capillary column, 50 m × 0.25 mm I.D., coated with trixylenyl phosphate (TXP). Nitrogen carrier gas inlet pressure: 5 p.s.i.g. Operating temperature: linearly programmed from 35° at 3°/min.

(iii) A column, similar to (ii) but coated with poly(phenyl ether), OS124 (PPE). Nitrogen carrier gas inlet pressure: 5 p.s.i.g. Operating temperature: linearly programmed from 35° at 2°/min.

TABLE I

RELATIVE RETENTION TIMES OF FATTY ACID METHYL ESTERS

Methyl ester of	*Boiling point**	*Relative retention time*			
		SE30	*TXP*	*PPE*	*DEGS*
Formic acid	32	1.51	2.73	—	0.86
Acetic acid	57	2.19	3.29	2.39	1.20
Propionic acid	80	3.35	3.76	3.06	1.64
iso-Butyric acid	92	4.12	4.10	3.42	1.86
n-Butyric acid	102	4.67	4.67	4.03	2.44
Tri-methylacetic acid	101	4.72	4.24	3.59	1.77
(±)-2-Methylbutyric acid	116	5.50	5.15	4.54	2.80
3-Methylbutyric acid	117	5.50	5.21	4.67	3.01
3,3-Dimethylbutyric acid	126	6.07	5.62	4.95	3.52
n-Pentanoic acid	127	6.08	6.08	5.52	3.88
2,2-Dimethylbutyric acid	127	6.24	5.62	4.95	3.20
(±)-2,3-Dimethylbutyric acid	136	6.55	6.20	5.53	3.88
2-Ethylbutyric acid	136	6.70	6.39	5.66	3.88
(±)-2-Methylpentanoic acid	138	6.86	6.39	5.94	4.16
(±)-3-Methylpentanoic acid	142	6.95	6.74	6.23	5.01
4-Methylpentanoic acid	144	7.09	6.90	6.42	5.20
n-Hexanoic acid	150	7.48	7.40	7.03	5.88
(±)-2-Methylhexanoic acid	157	8.06	7.66	7.32	6.18
(±)-3-Methylhexanoic acid	163	8.12	7.93	7.56	6.75
(±)-4-Methylhexanoic acid	161	8.55	8.41	8.25	7.32
5-Methylhexanoic acid	165	8.36	8.20	8.03	7.15
n-Heptanoic acid	173	8.78	8.75	8.60	7.92
n-Octanoic acid	193	10.00	10.00	10.00	10.00

* Approximate boiling point in °C at atmospheric pressure.

(iv) A support-coated open-tubular capillary column, 15 m × 0.5 mm I.D. coated with diethylene glycol succinate polyester (DEGS). Nitrogen carrier gas inlet pressure. 1 p.s.i.g. Operating temperature: linearly programmed from 35° at 1.5°/min.

The SE30 column was prepared in this laboratory according to a recently published method[9] and was somewhat shorter than the other open-tubular columns which were standard lengths supplied and coated by Perkin-Elmer Ltd.; the support-coated column was also supplied by Perkin-Elmer.

Results and discussion

Retention data for linearly temperature programmed analyses on four different stationary phases are detailed in Table I. All retention times were measured from sample injection and are presented as retention times relative to methyl *n*-octoate = 10.00.

The relative retention of times *n*-butyric ester, iso-butyric ester, *n*-pentanoic ester, *n*-hexanoic ester and *n*-heptanoic ester were almost identical on SE30 and TXP although the order of elution was not the same.

The order of elution on SE30 was largely determined by the boiling point of the esters but on the more polar stationary phases, especially DEGS, the order in which the compounds appeared was much influenced by their structure. On all stationary phases investigated, it was noted that 5-methylhexanoic ester was eluted before 4-

methylhexanoic ester; this appears to be anomalous on the basis of both the structures and (reported) boiling points of the two compounds.

The mixture was not wholly resolved on all the columns used. 2-Methylbutyric ester and 3-methylbutyric ester were coincident on SE30, as were 3,3-dimethylbutyric ester and *n*-pentanoic ester; both pairs of esters were resolved on the other stationary phases.

3,3-Dimethylbutyric ester and 2,2-dimethylbutyric ester were coincident on both TXP and PPE, but were separated on SE30 or DEGS.

2-Ethylbutyric ester and 2-methylpentanoic ester appeared together on TXP only.

n-Pentanoic ester and 2,3-dimethylbutyric ester were coincident on PPE and both were inseparable from 2-ethylbutyric ester on DEGS; all three compounds were resolved on TXP or SE30.

The retention characteristics of the DEGS support-coated column differ from those of the conventional open-tubular column with a similar number of theoretical plates (the latter being approximately three times the length of the former). The retention times of esters on the DEGS column were much shorter than on any of the conventional capillary columns, regardless of polarity.

However, this in no way detracted from the usefulness of this column when used in conjunction with other standard capillary columns for the identification of the compounds above.

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CHROM. 3688

Gas–liquid chromatography of diketopiperazines

Dipeptides have been separated by a variety of chromatographic procedures, including GLC of their N-trifluoroacetyl methyl esters[1–4]. Separation of dipeptides into diastereoisomers has been observed during paper[5] and thin-layer[6] chromatography, and also by GLC[1,3,4]; the latter observation is the basis for a method[3] for optical resolution of amino acids by GLC via dipeptide formation. In contrast, the chromatography of *cyclic* dipeptides (diketopiperazines) has received little attention, although an excellent thin-layer chromatographic system has been described[7]. Only one limited report[2] on GLC has appeared, in which a silicone column gave strongly asymmetric peaks and no separation of diastereoisomers. In the work reported here, symmetrical peaks were obtained with good diastereoisomeric separation in most cases. Application of the method to an investigation of diketopiperazine formation during thermal degradation of peptides will be described elsewhere.

Apparatus

A Glowall model 310 gas chromatograph equipped with a flame ionization detector and a Honeywell recorder was used, with coiled glass columns as follows: column A, 6 ft. × 3.4 mm of 3 % EGSP-Z on Gas Chrom Q, 100–120 mesh; column B, 3 ft. × 3.4 mm of 3 % SE-30 on Gas Chrom Q, 100–120 mesh. Carrier gas: argon at 40 ml/min.

Materials

The diketopiperazines containing alanine and phenylalanine were kindly supplied by WESTLEY*. Others were synthesised by published methods[8]; details of previously unknown compounds will be reported elsewhere. In some cases, diastereoisomeric mixtures were prepared from the *cis* (L-L) isomer by equilibration for 30 min in boiling 0.3 *N* methanolic sodium methoxide, a procedure which afforded mixtures containing mainly the *trans* isomer. Most of the N-methyl compounds were prepared by methylation[9] of the requisite parent diketopiperazine. Methanol or dimethylformamide were used as solvents for injection.

N,N'-Bis(trimethylsilyl) (TMS) derivatives[10] were typically prepared as follows: the diketopiperazine (1–5 mg) in a 25 % solution (0.2–1.0 ml) of N,O-bis(trimethylsilyl) acetamide in dry dimethylformamide was heated for 10 min at 80° in a capped vial. An aliquot of the resulting solution, which contained excess reagent, was injected directly into the gas chromatograph.

Results

Retention times of various diketopiperazines on column A at two temperatures, and of the relatively volatile N-methyl compounds at the lower temperature, are given in Table I. Narrow, symmetrical peaks were obtained and essentially similar separations were observed with a column of 1 % EGSP-Z on Gas Chrom P, on which retention times were approximately halved. A 3 % NGS column was inferior for this purpose.

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TABLE I

RETENTION TIMES (min) OF DIKETOPIPERAZINES ON COLUMN A

Diketopiperazine	*206°*	*196°*	*N-Methyl-diketopiperazine*	*196°*
Gly-Gly	25.3	41.5	Sar-Sar	5.8
Gly-Ala	15.5	25.6	Sar-Val	9.5
Gly-Val	17.1	28.7	Sar-Meval	5.3
Gly-Pro	22.8	37.5	Sar-Pro	13.8
Ala-Val (*c*+*t*)	10.4	17.4	Sar-Cycloleu[a]	17.2
Ala-Leu (*c* + *t*)	13.4	22.1	Val-Meval (*c*)	10.5
Val-Val (*c*)	10.6	17.2	Val-Meval (*t*)	7.0
Val-Val (*t*)	9.6	15.4	Meval-Meval (*c*)	5.4
Val-Pro (*c*)	12.3	19.5	Meval-Meval (*t*)	3.5
Val-Pro (*t*)	16.7	26.8		
Ileu-Pro (*c*)	16.3	25.7		
Ileu-Pro (*t*)	21.8	35.2		

[a] "Cycloleu" refers to aminocyclopentane-1-carboxylic acid[11].

TABLE II

RETENTION TIMES OF TMS-DIKETOPIPERAZINES ON COLUMN B

Diketopiperazine	*Temperature*	*Retention time*
Gly-Gly	115°	4.1
Ala-Val (*c*)	115°	5.4
Ala-Val (*t*)	115°	4.9
Ala-Leu (*c*+*t*)	115°	10.6
Val-Val (*c*)	115°	11.3
Val-Val (*t*)	115°	9.7
Gly-Phe	165°	4.5
Ala-Phe (*c*)	165°	4.9
Ala-Phe (*t*)	165°	4.5
Leu-Phe (*c* + *t*)	165°	8.0

Column A gave a base-line separation of the *cis* (L-L or D-D) and *trans* (D-L) forms of valine diketopiperazine. An even higher degree of diastereoisomeric resolution was observed for valyl-prolyl and the structurally similar isoleucyl-prolyl diketopiperazines; retention time ratios (*trans*/*cis*) were 1.37 in each case. In contrast, this column failed to distinguish the diastereoisomers of alanyl-valyl and alanyl-leucyl diketopiperazines.

Retention times of some N-trimethylsilyl diketopiperazines on column B are given in Table II. The diketopiperazines containing phenylalanine required a higher temperature for GLC than the purely aliphatic ones.

Discussion

On the EGSP-Z column the diketopiperazines under study displayed a wide range of retention times, shortest for those containing alanine or valine and longest for those in which glycine or proline was present. N-Methylation drastically reduced retention times. The degree of diastereoisomeric separation varied greatly, being

highest for diketopiperazines containing proline or N-methylvaline and zero for those containing alanine.

N-Trimethylsilylation permitted the satisfactory use of a column of the silicone type, on which retention times were comparatively dependent upon molecular weight. Diastereoisomeric separation was again encountered, but for the diketopiperazines composed of simple aliphatic amino acids the use of column A without derivatization was generally superior. For less volatile diketopiperazines, however, the advantage of trimethylsilylation is obvious, since it permits GLC on silicone columns without "tailing". The diketopiperazines containing phenylalanine could not be chromatographed on column A, but on column B, after trimethylsilylation, convenient retention times were obtained at a temperature far below maximum. It follows that GLC may be applicable to even less volatile diketopiperazines by this means, since additional functional groups would also be trimethylsilylated. This possibility is under experimental study.

Applications of the techniques described here in the areas of peptide chemistry and natural products are under investigation.

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A method for gas chromatographic determination of benzodiazepines

It has been recently reported by RUELIUS *et al.*[1] and by SCHWARTZ *et al.*[2], that diazepam in man and dog undergoes N-demethylation and C_3-hydroxylation. The metabolites produced were characterized by SCHWARTZ *et al.*[3] by combining thin-layer chromatography with high-resolution mass-spectrometry.

A gas chromatographic method for the determination of diazepam and its demethylated metabolites, involving an acidic hydrolysis to chlorobenzophenone derivatives, was described by DE SILVA *et al.*[4]. This method, although later modified (DE SILVA *et al.*[5]), permits a separation between diazepam and its N-demethylated metabolites, but does not allow differentiation of the C_3-hydroxylated metabolites.

An improved gas chromatographic procedure is described here which employs a liquid phase of 3 % OV_1. This gives an excellent resolution and allows a quantitative estimation of the individual intact benzodiazepine derivatives. The present method has been successfully applied to the determination of diazepam, N-demethyl-diazepam, N-methyl-oxazepam, oxazepam and nitrazepam.

Experimental

Reagents. All reagents must be of reagent grade purity. All inorganic reagents were made up in triple distilled water.

1 M KH_2PO_4 buffer: pH 7.

Diethyl ether: Analytical reagent grade ether, containing not more than 0.00005 % peroxide, must be used from a bottle opened no more than 1 day previously.

Acetonitrile: Reagent grade pure for spectrophotometric use.

Procedure

In a glass-stoppered centrifuge tube place 1 ml of the sample containing benzodiazepine derivatives, 2 ml of buffer and 4 ml of H_2O. Add 10 ml of ether, seal the stopper with water and shake on a reciprocating shaker for 10 min. Centrifuge at 0° for 5 min and transfer the ether phase into a glass tube. Re-extract the water phase with another 10 ml of ether and combine the ether extracts. Place the tube containing the ether extracts in a hot water bath (45°) to evaporate to dryness and then dry the residue in a vacuum desiccator for 15 min. Dissolve the residue in a suitable amount of acetonitrile and ensure uniform distribution and solution of the material by tapping and stirring the tube for 60 sec.

A suitable aliquot, from 1 to 3 μl, is chromatographed and the different peaks are identified by their retention times.

Gas chromatography. The gas chromatograph used was Model G.V. dual column (Carlo Erba, Milan) equipped with a hydrogen flame ionization detector. In several experiments an electron capture detector was used to improve the sensitivity of the method.

The stationary phase was OV_1 3 % on Gas Chrom Q (60–80 mesh) packed into a 2-m glass column (int. diam. 2 mm, ext. diam. 4 mm). The flow rate of carrier gas (nitrogen) was 22 ml/min and the column temperature was 245°.

Quantitative analysis. For identification and calculations the internal standard

technique was used. 2-N-Benzylamino-5-chloro-benzophenone 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.

Benzodiazepines can be quantitated by gas chromatography when the relative peak area is used as an index of concentration, since a linear relationship exists between relative peak area and drug concentration in the range of 0.1 to 2 μg.

A hundred-fold increase in sensitivity was obtained by using the electron capture detector.

Preliminary studies of the partition coefficient of benzodiazepines tested between water and ether have shown that these drugs are substantially quantitatively extracted from aqueous media with a 2-fold volume of this organic solvent. A typical gas chromatogram showing the separation of a mixture containing five benzodiazepine derivatives and the internal standard is illustrated in Fig. 1.

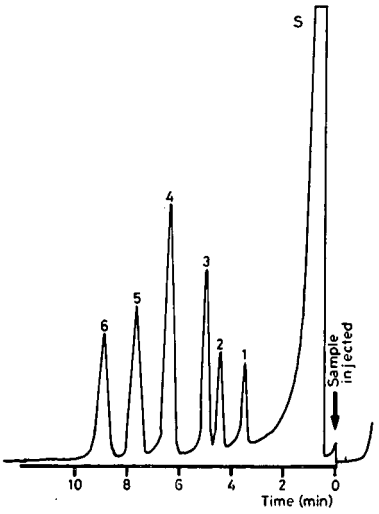

Fig. 1. Separation of a mixture of five benzodiazepines. 1 = Oxazepam; 2 = diazepam; 3 = N-demethyl-diazepam; 4 = N-methyl-oxazepam; 5 = nitrazepam; 6 = internal standard; S = solvent. Experimental conditions as described in the text.

TABLE I

RECOVERY STUDIES

Drug	*% Recovery ± S.E.*	
	From water	*From rat blood*
Diazepam	92 ± 3	90 ± 3
N-Demethyl-diazepam	100 ± 4	100 ± 3
N-Methyl-oxazepam	93 ± 1	65 ± 2
Oxazepam	91 ± 1	63 ± 2
Nitrazepam	96 ± 2	87 ± 3

The recovery from water and from rat blood is reported in Table I. The lower recovery from blood suggests a possible protein-binding of the hydroxylated derivatives.

Preliminary studies indicate that blanks from extracts of rat blood or tissue do not show peaks that interfere seriously with a biological application of the gas chromatographic procedure.

The method described in this paper has the advantage over the previous methods of being simple and rapid. It also permits measurements of diazepam and its metabolites in the same sample.

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An accurate siphon for measuring fractions during column chromatography

A number of commercial fraction collectors employ a counterbalanced arm, which causes the turntable to rotate as it rises and falls. The arm is operated by the filling and emptying of a siphon attached to one end. This is a convenient system but the uniformity of the fractions produced depends entirely on the consistency of the siphon. The only form of siphon available commercially, as far as I know, is of type A1 (Fig. 1). Devices of this sort were described by NEDERBRAGT[1] and LIGON[2] but were first studied as an aid to column chromatography by BOVÉ[3]. Modifications intended for special purposes have since been reported[4,5] but BOVÉ's basic design has remained

unchanged. I have used several siphons of this type corresponding to the original specification[3] in essential details and these have all yielded relatively inconsistent results (see Table I).

There are two reasons for the poor performance of type A siphons. First, after each delivery, liquid will be left adhering to the walls of the siphon tube (d). This liquid tends to coalesce into a broken column which is partially displaced to the descending limb of the siphon tube as the main vessel (f) fills, and causes premature siphoning by exerting a small hydrostatic pressure downwards. The effective length of this column, and hence the error induced, varies from one delivery to another.

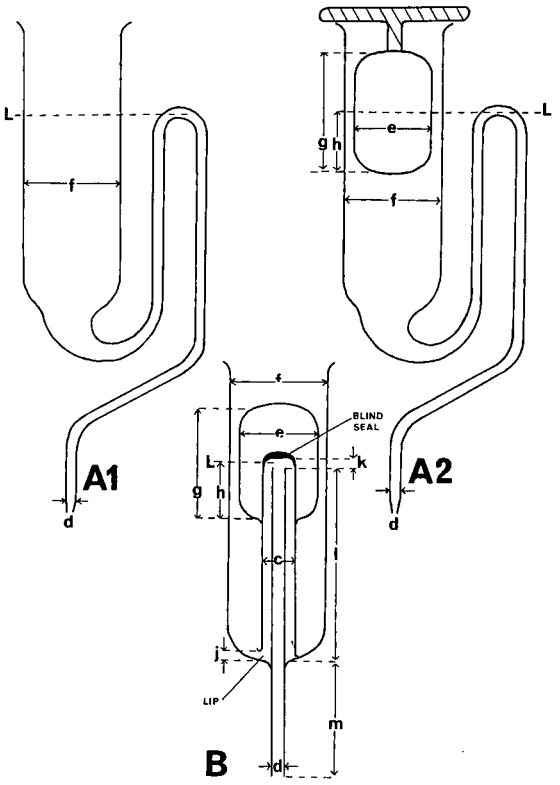

Fig. 1. Types of siphon investigated. In the text, each tube is identified by the letter used to indicate its diameter in these drawings.

This phenomenon is more noticeable with water than it is with liquids of lower surface tension such as acetone, and this is why acetone is siphoned slightly more consistently than water (Table I). This source of variation may be minimised by rigorous cleaning of the siphon, since the walls of a chemically clean siphon tube will drain away residual liquid so rapidly that it usually does not coalesce. In my experience, very rigorous cleaning agents are necessary; soaking in aqua regia (see Table I for conditions) is effective and does improve the consistency of type A siphons (Table I). Nevertheless, the 5 ml siphon tested, after cleaning in aqua regia, still

TABLE I

CONSISTENCY OF SIPHONS

For each test, the siphon was supplied with the solvent stated at a steady flow rate in the range 0.7–1.2 ml/min. Twenty consecutive fractions were collected and weighed to an accuracy of ± 5 mg. The maximum variation and standard deviation of each such group of results is given as a percentage of the average weight of the fractions delivered during that run.
Cleaning procedure: siphons were either (i) rinsed six times in the solvent being used or (ii) soaked for 24 h in 12 *N* hydrochloric acid–concentrated nitric acid (3 : 1, v/v), rinsed six times in tap water and then six times in the relevant solvent.

Type of siphon and nominal capacity	*Cleaning procedure*	*Solvent used*	*Maximum variation observed (20 deliveries)*	*Standard deviation (20 deliveries)*
A1, 5 ml	Rinsed	Water	±27.7%	±13.9%
	Aqua regia	Water	±13.0%	± 7.0%
	Rinsed	Acetone	±15.5%	±10.3%
A2, 5 ml	Aqua regia	Water	± 4.3%	± 2.7%
B, 1 ml	Rinsed	Water	± 1.2%	± 0.6%
B, 5 ml	Rinsed	Water	± 2.4%	± 1.0%
	Rinsed	Ethanol	± 0.3%	± 0.2%
	Rinsed	Acetone	± 1.9%	± 0.8%
	Rinsed	Xylene	± 0.6%	± 0.3%
B, 100 ml	Rinsed	Water	± 1.5%	± 0.9%

allowed a broken column of water to form twice during the first twenty operations, so such cleanliness only partially solves the problem. However, in the next paragraph, to simplify description, I shall temporarily assume that this source of variation has been eliminated.

The second source of error in type A siphons is the residual liquid left at the tip of the siphon tube. This will be bound by capillarity and form a liquid seal which must be displaced before siphoning can occur. The liquid in the main vessel does not therefore siphon over at level L but continues to rise until it exerts sufficient excess pressure to blow out the liquid seal. The level at which siphoning actually occurs varies from one operation to another, and this is the main source of inconsistency in clean siphons. This problem should not arise if the diameter of the siphon tube is increased towards its mouth as in some commercial siphons. However, this second source of error is unavoidable if the siphon tube contains much residual liquid from the previous delivery; *i.e.* the first source of inconsistency is always compounded with the second.

However, there is a very easy way of minimising the errors from both sources. If the cross-sectional area of the main siphon vessel is reduced, variations in the level at which siphoning occurs will cause smaller errors in the volumes delivered. For example, siphon A2 (Fig. 1), which has a cross-sectional area in the relevant region 33 % of that of siphon A1, gives a standard deviation for water lower by a factor of 2.6 (Table I). Siphon A2 is identical to A1 except that a sealed tube (e), supported by a T piece, has been inserted into the main vessel. The difference between the external diameter of tube e and the internal diameter of tube f must be at least 0.15 cm if liquid is to run in freely. Though the reduction in cross-sectional area which may be

obtained in this way is limited, this is a convenient way of improving the performance of an existing type A siphon.

However, the main purpose of this account is to describe a new siphon which automatically blows residual liquid out of its siphon tube between each delivery and so avoids all the sources of inconsistency found in the conventional siphon. This modification, type B in Fig. 1, operates as follows. After each delivery, about two-thirds of the space between tubes c and d will be full of liquid and there will be the usual broken column present in the siphon tube, d. As the main vessel, f, fills up, liquid flows through the lip and displaces air from the top of c. The air, in turn, drives residual liquid down d and, by the time the level in the main vessel reaches the constricted region, tube d will be empty except for a single drop held at its tip by capillarity. The incoming liquid rises rapidly in the narrow space between e and f and its pressure is soon sufficient to blow out this last drop. More liquid is now free to pass into c, the level in the main vessel falls slightly, and siphoning will occur at level L. Siphoning ceases when air is drawn into c through the lip.

The blind seal at the end of tube c is essential to the success of this type of siphon. In its absence, organic liquids and sometimes even water, will continuously trickle down the internal wall of the siphon tube, instead of siphoning over as a solid column, and so defeat the whole purpose of the device. The blind seal prevents this by providing a plate of glass immediately above the entrance to the siphon tube. Liquid clings to this plate; its meniscus is therefore presented to the siphon tube from above, not from below as previously, and this initiates correct siphoning.

In order to prove the complete reliability of B type siphons, the 5 ml version was used to deliver 550 fractions of aqueous buffer and, later, 1104 fractions of acetone, which is particularly liable to cause siphon failure in the way just described because of its low viscosity and surface tension. Flow rates were 0.3 ml/min and 1 ml/min, respectively. The siphon operated perfectly throughout, and there was no failure of any kind.

Type B siphons, as expected, do give consistent results with both water and organic solvents (Table I). Indeed their performance with ethanol is remarkable since the residue left in the 5 ml siphon after each delivery (about 0.7 ml) cannot have varied by more than $\pm$ 15 μl during the test period. Consistency is maintained over long periods. The 5 ml siphon has been used to receive the aqueous effluent from an ion exchange column used for separating amines, each separation taking three days and requiring the collection of 300 fractions. During five runs, the relative position of the ammonia peak in the series of fractions only varied by $\pm$ 0.9 % and that of the tyramine peak by $\pm$ 0.5 %. Moreover, the siphon would only have been responsible for part of these small variations. The most valuable feature of type B siphons is their ability to give such consistent results without special cleaning and, indeed, without attention of any sort. The mean volume delivered by one of these siphons should be independent of the liquid being used. Mean volumes delivered by the 5 ml siphon were: water, 4.91 ml; ethanol, 4.82 ml; acetone, 4.84 ml; xylene, 4.90 ml; variation, $\pm$ 0.045 ml, *i.e.* $\pm$ 0.9 %.

B type siphons are more compact, possibly slightly more robust, than the conventional sort and they deliver their liquid rather more rapidly. Emptying times for the siphons investigated, measured with water, were: 5 ml A1, 9 sec; 1 ml B, 2.5 sec; 5 ml B, 7 sec; 100 ml B, 35 sec.

The one disadvantage of B type siphons is that they retain a considerable volume of residual liquid after each delivery, most of it between tubes c and d. This residue is efficiently siphoned over during the next operation but, inevitably, represents a transfer of material from the first fraction to the succeeding one. The magnitude of this cross contamination is given in Table II. This factor is usually of little practical importance, but with small siphons (1 or 2 ml) and a column effluent containing comparatively sharp peaks, it will cause appreciable distortion of these peaks. Attempts to reduce the degree of cross contamination by progressively decreasing the volume between tubes c and d, lead, first, to inconsistent deliveries, and finally, to a siphon which will not operate at all. In some circumstances an A type siphon, which causes very little cross contamination, may be a better compromise.

Type B siphons delivering less than 1 ml are very difficult to construct and will probably never be useful, but there are no other obvious size restrictions. Siphons made to the dimensions given in Table III will usually deliver somewhat less than the nominal volume indicated. The delivery volume is most conveniently adjusted to the exact value required by blowing out the lower part of the side wall of tube f. In designing this type of siphon, the following limitations should be observed. The internal

TABLE II

CROSS CONTAMINATION OF FRACTIONS DUE TO USE OF SIPHONS

Each siphon tested was rinsed, then completely filled, with 1.0% aqueous potassium dichromate. The siphon was then supplied with pure water at a flow rate of 1 ml/min, and the dichromate present in each of the fractions was determined colorimetrically at 495 mμ. The figures given represent the concentration of dichromate in fractions 2, 3 and 4, taking the concentration of solute in fraction 1 (the original solution) as 100%.

Type of siphon and nominal capacity	*Percentage of solute transferred from fraction 1 to:*		
	Fraction 2	*Fraction 3*	*Fraction 4*
A1, 5 ml	2.9%	> 0.1%	—
A2, 5 ml	3.9%	> 0.1%	—
B, 1 ml	39.5%	18.8%	> 0.1%
B, 5 ml	14.2%	1.4%	> 0.1%
B, 100 ml	2.6%	> 0.1%	—

TABLE III

DIMENSIONS OF SIPHONS TESTED

The siphons were all constructed of borosilicate glass and dimensions are given in centimetres. Int = internal diameter; Ext = external diameter.

Type and nominal capacity	*c (Int)*	*d (Ext)*	*d (Int)*	*e (Ext)*	*f (Int)*	*g*	*h*	*j*	*k*	*l*	*m*
A2, 5 ml			0.18	0.80	0.98	4.0	2.0				
B, 1 ml	0.48	0.38	0.20	0.63	0.83	4.0	2.0	0.25	0.20	4.8	4.2
B, 5 ml	0.53	0.38	0.20	0.99	1.14	4.0	2.0	0.2	0.15	7.2	3.9
B, 100 ml	0.86	0.64	0.25	2.96	3.28	4.0	2.0	0.3	0.20	12.3	11.5

diameter of tube d should be in the range 0.2–0.25 cm; if smaller than this, liquid is held in the bore too strongly by capillarity and may not be blown out properly between deliveries; if larger there will be some risk of siphon failure due to liquid trickling continuously down the wall. A siphon tube of 0.4 cm internal diameter has been successfully used to obtain a high delivery rate when siphoning water, but a tube of this size is only suitable for special purposes. Dimension l may be adjusted to any convenient value and dimension m may be varied from 4 to 11 cm. The volume of the space between tubes c and d should be approximately 2.5 times the volume of the bore of tube d, to ensure that this is thoroughly blown out between deliveries. However, any increase in the ratio of these volumes will augment the amount of cross contamination caused by the siphon. Tube f may be of any convenient size but the difference between the internal diameter of tube f and the external diameter of tube e should not be less than 0.15 cm if liquid is to flow freely into the siphon. Increasing the clearance beyond this causes some loss of consistency. In siphons delivering less than 5 ml, tube c will be too large to fit inside e. The blind seal must then be made in tube e, at the appropriate level. Dimensions g and h are not critical, and the values given may be altered by $\pm$ 0.3 cm. Dimension k should be in the range 0.05–0.2 cm to ensure that the blind seal really induces correct siphoning. Finally, the lip should be an approximately square indentation in the wall of tube c, having an area of about 5 sq. mm. If the lip is too small, it will not allow air to be drawn into tube c at the end of the first delivery; from then on the siphon will be useless, because incoming liquid will be siphoned over continuously, instead of being delivered in accurately measured portions. A very large lip should also be avoided since, after siphoning has ceased, this allows the residual liquid from tube c to run back into tube f, and increases the extent to which the first fraction mixes with the succeeding ones.

This type of siphon is certainly well worth constructing, since it will give very consistent results with water and organic solvents, yet, unlike the conventional siphon, needs no special cleaning.

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Fractionation of *Escherichia coli* DNA using poly-L-lysine Kieselguhr columns

Fractionation of DNA from complexes formed with poly-L-lysine has been reported previously[1], with increasingly concentrated salt solutions resulting in the separation of differently composed nucleic acid fractions. Recent work in our laboratory has shown that the main basis for the fractionation of the DNA–polylysine complex is that of base composition, unlike various other fractionation techniques recently reported, dependent on either molecular weight or hydrogen bonding properties or both[2]. The fractionation has been developed, using poly-L-lysine supported on Kieselguhr columns, loading with DNA, and determining the continuous elution profile of the columns with a linear gradient of sodium chloride from 0.4 *M*–4.0 *M*[3]. This communication reports the stepwise fractionation of DNA isolated from *Escherichia coli* strain B.

DNA was isolated using the method of MARMUR[4]. Poly-L-lysine Kieselguhr columns were set up as described previously[3]. DNA at a concentration of 100 μg/ml (15 ml) in 0.4 *M* NaCl/0.02 *M* KH_2PO_4 buffer was loaded onto the column under air pressure of 2 lb./sq.in. The column was eluted in a stepwise fashion with increasingly concentrated solutions of sodium chloride from 1.0 *M* to 4.5 *M*. Thirty millilitres of each concentration were found to be sufficient to complete the elution of a DNA peak. The flow rate was adjusted to about 20 ml/h, and 3 ml fractions were collected. The DNA in each fraction was determined from the extinction at 260 mμ. The elution profile is summarised in Fig. 1.

It is seen from Fig. 1 that the *E. coli* DNA was fractionated into four peaks, eluted at 1.0 *M*, 1.5 *M*, 2.5 *M* and 3.0 *M* NaCl. The fractions of each peak were combined, exhaustively dialysed against distilled water at 2°, and adjusted to an optical density of about 0.2 at 260 mμ with distilled water. They were then assayed

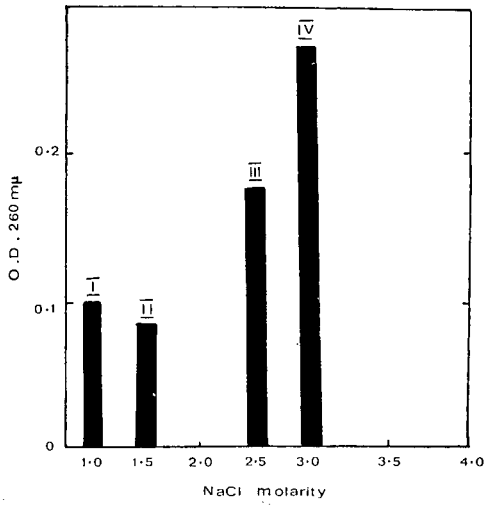

Fig. 1. Stepwise elution profile of the effluents from a PLK column, previously loaded with native *E. coli* DNA (1.5 mg in 15 ml 0.4 *M* NaCl buffer), and eluted with sodium chloride solutions of increasing concentrations from 1.0 *M* to 4.5 *M* (see text).

for base composition using the method of FREDERICQ, OTH AND FONTAINE[5]. The results are shown in Table I. The base composition of native DNA before fractionation when analysed by this method was 52 % GC.

TABLE I

BASE ANALYSIS OF DNA FRACTIONS ELUTED FROM PLK COLUMNS

Peak No.	*Salt molarity eluted*	*% GC*
I	1.0 *M*	53
II	1.5 *M*	40.5
III	2.5 *M*	45.5
IV	3.0 *M*	64

It can be seen from Table I that each of the peaks has a different average base composition. Peak I has a % GC of 53 which is very close to that of native, unfractionated DNA. Peaks II and III have a % GC substantially lower than native DNA, while that of peak IV is substantially higher than native DNA. It is also noted that the % GC of the peaks increases with salt concentration from peak II to peak IV, DNA with low GC content eluting with lower NaCl concentration.

The results obtained using the PLK column were compared with those obtained by extraction of a poly-L-lysine–DNA complex with increasingly concentrated sodium chloride solutions. 2.0 mg DNA in 15 ml 0.02 *M* KH_2PO_4/0.4 *M* NaCl buffer (pH 6.7) was mixed with 1.6 ml polylysine (10 mg/ml). A precipitate which was formed immediately was centrifuged at 12,000 g for 10 min, and the pellet washed twice with 0.4 *M* NaCl buffer. The pellet was suspended in 15 ml 1.0 *M* NaCl using a Potter homogeniser, and then centrifuged at 12,000 *g* for 10 min. This procedure was repeated twice at each concentration of sodium chloride. This technique did not give an exhaustive extraction, but gave DNA fractions suitable for qualitative evaluations. The fractions at each concentration were combined. Analysis of DNA content, and base composition of the fractions was carried out as described above. The results are summarised in Table II.

The essential similarity of this extraction pattern to that obtained on elution

TABLE II

BASE ANALYSIS OF DNA FRACTIONS EXTRACTED FROM DNA–POLYLYSINE COMPLEXES

Concentration of NaCl	*O.D. at 260 mμ*	*Base content (% GC)*
1.0 *M*	0.142 (peak I)	51
1.5 *M*	0.135 (peak II)	43
2.0 *M*	0.008	—
2.5 *M*	0.193 (peak III)	47
3.0 *M*	0.387 (peak IV)	60
3.5 *M*	0.020	—
4.0 *M*	0.030	—

from a PLK column is noted. This serves to confirm the validity of DNA fractionation on PLK columns, and also to emphasize the convenience engendered by the use of a column.

The results presented here indicate that DNA from *Escherichia coli* strain B is fractionated into peaks of different base composition on poly-L-lysine Kieselguhr (PLK) columns, DNA with low GC content eluting at low NaCl concentrations, and DNA with higher GC content eluting with increasingly concentrated NaCl solutions. These results serve as a confirmation of those obtained previously[3], using DNA from the Marburg strain of *Bacillus subtilis*.

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CHROM. 3651

Characterization of fiber parameters in agarose gels by light scattering

Gel chromatography has been proposed as a method for determining the fiber parameters of gels. Using this technique, the structures of dextran gels, a hyaluronic acid gel, agarose gels and an elastin gel have been postulated[1–5]. However, only in the case of hyaluronic acid has it been possible to confirm the results by an independent technique, namely, electron microscopy[6].

In this communication, light scattering data on agarose will be reported. They have been evaluated as proposed by CASASSA[7], and corroborate the parameters obtained by gel chromatography.

Experimental

Two different preparations of agarose were used. One was prepared according to HJERTÉN[8], and the other was a pearl-condensed agarose gel, kindly supplied by Dr. B. GELOTTE, Pharmacia, Uppsala. From these two batches, the following concentrations of agarose were prepared: 10×10^{-4}, 9×10^{-4}, 8×10^{-4}, 6×10^{-4}, 5×10^{-4}, 4×10^{-4}, 2×10^{-4} and 1×10^{-4} g/ml. All solutions were made up in 0.2 *M* NaCl.

The solutions were warmed to 90° for 18 h and then centrifuged in glass cells at 18,000 r.p.m. for 20 min in a Spinco Model L preparative ultracentrifuge using rotor SW 25, as described by DANDLIKER AND KRAUT[9]. The temperature of the rotor was 60–70°. This procedure cleared the solutions from dust.

After centrifugation, all solutions were allowed to stand at room temperature for 48 h, in order that the agarose can polymerize. The angular distribution of the scattered light at a wavelength of 4360 Å was then measured at 40°–130° in a Brice-Phoenix light scattering photometer using the same cells as those in which the centrifugations were performed. The photometer was calibrated with Ludox according to MARON AND LOU[10]. As agarose is a polygalactose, a value of the refractive increment

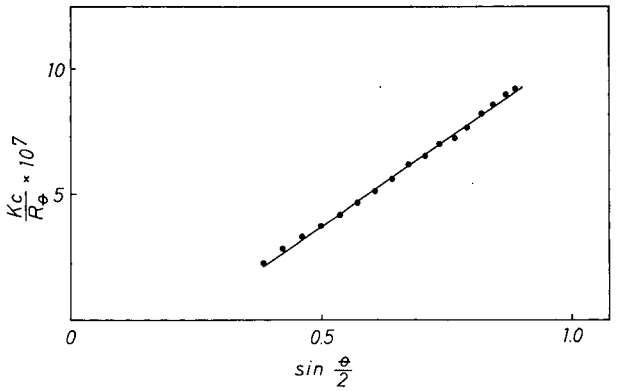

Fig. 1. Plot according to CASASSA[7] of light scattering data obtained on 6×10^{-4} g/ml agarose. ($K = 2\pi^2 n^2 (dn/dc)^2 / N\lambda^4$, where n is the refractive index of the solvent; dn/dc the refractive increment, 0.152 ml/g; N = Avogadros number and λ the wavelength of the light. c is the concentration of agarose in g/ml and R_θ is the reduced angular intensity measured at the angle θ). From the slope of the line, one can calculate a mass/length ratio of 1.72×10^{-13} g/cm.

of 0.152 ml/g was used. This corresponds to the refractive increment of dextran[11]. The refractive increments of other polyhexoses are in the same range[12].

The light scattering data were treated according to CASASSA (Fig. 1). A plot of Kc/R_θ *vs.* $\sin \theta/2$ should be linear for a system of randomly distributed fibers, and the slope of the line should be related to M/L, *i.e.* the mass per length unit of the fibers. The M/L values for agarose determined in this way for the concentrations 10×10^{-4}–4×10^{-4} g/ml are in the range 1.26–1.72×10^{-13} g/cm. Concentrations below 4×10^{-4} g/ml did not give straight lines, and hence no M/L-values could be calculated at these concentrations. This might depend on a less uniform fiber structure at these low concentrations. The corresponding values obtained from gel chromatography[4] are 1.19–1.69×10^{-13} g/cm and are in very good agreement with the light scattering results. It should be stressed that the concentrations of agarose in the gel chromatographic experiments, 4×10^{-2}, 6×10^{-2} and 8×10^{-2} g/ml respectively were much higher than the concentrations in the light scattering experiments. Light scattering could not be performed on more concentrated gels, due to the high turbidity.

Although CASASSA predicted that the line in Fig. 1 should pass through the origin, it is evident from the diagram that this is not the case. This can, however, be explained if it is assumed that a small number of very large aggregates are present in the gel. The slope of the line would not, on this assumption, be significantly changed. In experiments with fibrin, CASASSA found similar negative intercepts on the Kc/R_θ-axis.

Using two different methods, gel chromatography and light scattering, similar values for the fiber parameters of agarose gels were obtained thus providing additional evidence in favour of the theory for gel chromatography proposed by LAURENT AND KILLANDER[1], and justifying the use of gel chromatography for characterizing gels.

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CHROM. 3705

Bacterial lipopolysaccharide as a void volume marker for agarose gel permeation chromatography

The current wide availability of agarose preparations for molecular exclusion chromatography has provided a convenient technique for the separation of high molecular weight macromolecules. The use of agarose preparations having exclusion limits above a molecular weight of 2×10^6 presents a problem in the determination of the void volume of the column, since there is no commercial marker available for such determinations.

The author has found that a preparation of lipopolysaccharide from Gram-negative bacteria serves as a convenient marker for the determination of the void volume of agarose columns with an exclusion limit of 1.5×10^8.

Materials and methods

Agarose gels. Columns were prepared using spherical beads of agarose obtained from Bio-Rad Laboratories (Richmond, Calif.) and from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). The Bio-Rad preparations included Bio-Gel A5, A15, A50 and A150, having molecular weight exclusion limits of globular materials of 5×10^6, 15×10^6, 50×10^6 and 150×10^6, respectively. Sepharose 2B, having an exclusion limit of 25×10^6, was the only Pharmacia preparation tested. The columns used were 2.5 cm in diameter, ranged from 30–45 cm, in length, and were packed in accordance with the instructions of the manufacturer. Both ascending and descending elution was used at various times. After packing, the columns were washed with one to two bed volumes of 0.05 *M* Tris(hydroxymethyl)aminomethane buffer, pH 7.2.

Lipopolysaccharide. For most of the work reported here, phenol–water extracts of *Salmonella typhimurium* were prepared as described by LÜDERITZ, STAUB AND WESTPHAL[1]. A similar preparation of *S. typhosa* 0901, obtained from Difco Laboratories (Detroit, Mich.), was also utilized.

Methods

Samples of 2 to 10 mg in a volume of 0.5 to 2 ml (less than 1% of the bed volume) were applied to the column and eluted with Tris buffer as described above. Fractions of 5 ml were collected volumetrically and each fraction assayed for carbohydrate by the phenol–sulfuric acid technique of DUBOIS *et al.*[2]. Tubes containing known amounts of lipopolysaccharide were interspersed throughout the series of collected fractions during assay to verify the accuracy of the procedure.

Results and discussion

The recovery of the lipopolysaccharide prepared in this laboratory was quantitative, with all the material eluting essentially in one peak at (approximately 25% of the bed volume) the expected void volume of the column (Fig. 1). The Difco preparation was found to yield two minor peaks following the major peak closely. These materials should not reduce the efficacy of the preparation as a void volume marker. The structure of lipopolysaccharide as shown by SHANDS *et al.*[3] and RUDBACH *et al.*[4] is that of a fibrillar network yielding an extremely high molecular diameter. The

final step in the purification of the lipopolysaccharide, centrifugation at 105,400 × *g* for 1 h, is essential to ensure that the material is eluted as a single peak. Crude extracts yield two peaks on high exclusion limit agarose preparations such as A150.

Since the DUBOIS procedure is a general technique for the detection of carbohydrate, the column must be washed with a minimum of two bed volumes of eluant prior to the loading of the column. Assay of eluant from columns left standing for several days showed small amounts of carbohydrate in the eluant, indicating the leaching of carbohydrate from the gel.

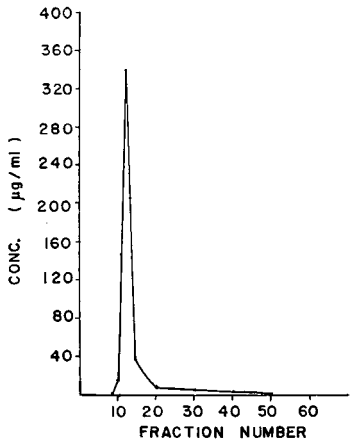

Fig. 1. A typical elution pattern of lipopolysaccharide from *S. typhimurium* on A150. The bed volume was 248 ml. A sample of 4 mg was applied to the column.

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CHROM. 3627

Paper chromatography of aza-heterocyclic hydrocarbons

III. Some further paper and thin layer systems

In two recent communications from this laboratory we have examined a series of paper chromatographic systems for the separation of aza-heterocyclic hydrocarbons[1,2]. As mixtures encountered in air pollution studies involve possibly hundreds of compounds numerous chromatographic methods have been employed and still others will be needed.

TABLE I

R_F VALUES OF AZA-HETEROCYCLICS ON PAPER DEVELOPED WITH AQUEOUS H_2SO_4

Paper: Whatman 3MM. Temperature: 18–20°. Eluant: aqueous H_2SO_4.

Substance	*0.1 N*	*0.5 N*	*1.0 N*	*2.0 N*	*3.0 N*
Acridine	0.77	0.74	0.73	0.71	0.71
2-Methylacridine	0.70	0.64	0.63	0.63	0.61
3-Methylacridine	0.70	0.64	0.64	0.62	0.61
Benz(*a*)acridine	0.25	0.23	0.21	0.18	0.17
Benz(*c*)acridine	0.35	0.32	0.26	0.26	0.25
8,12-Dimethylbenz(*a*)acridine	0.21	0.18	0.17	0.14	0.13
7,9-Dimethylbenz(*c*)acridine	0.10	0.09	0.12	0.05	0.05
8,10-Dimethylbenz(*a*)acridine	0.14	0.12	0.10	0.06	0.04
7,10-Dimethylbenz(*c*)acridine	0.11	0.09	0.10	0.08	0.05
8,10-Dimethylbenz(*c*)acridine	0.13	0.11	0.09	0.07	0.04
9,12-Dimethylbenz(*a*)acridine	0.13	0.10	0.12	0.09	0.09
Dibenz(*a,h*)acridine	0.01	0.01	0.00	0.00	0.00
Dibenz(*a,j*)acridine	0.00	0.01	0.00	0.00	0.00
Pyrenoline	0.06	0.04	0.04	0.03	0.02
Benzo(*c*)cinnoline	0.48	0.64	0.69	0.78	0.77
9-(2′-Pyridyl)-anthracene	0.30	0.27	0.25	0.20	0.17
9-(3′-Pyridyl)-anthracene	0.18	0.15	0.15	0.12	0.10
9-(4′-Pyridyl)-anthracene	0.16	0.12	0.13	0.09	0.07
10-(2′-Pyridyl)-1,2-benzanthracene	0.02	0.01	0.00	0.01	0.01
10-(3′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00	0.00	0.00
10-(4′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00	0.00	0.00
14-Phenyldibenz(*a,j*)acridine	0.00	0.00	0.00	0.00	0.00
7-Phenyldibenz(*c,h*)acridine	0.00	0.00	0.00	0.00	0.00
Acridone	0.11				0.17
Phenanthridine	0.69	0.70	0.66	0.65	0.65
1-Azapyrene	0.47	0.43	0.42	0.41	0.35
4-Azapyrene	0.48	0.43	0.42	0.39	0.36
Benzo(*h*)quinoline	0.74	0.70	0.71	0.70	0.68
Benzo(*f*)quinoline	0.70	0.65	0.64	0.64	0.61
3-Methylbenzo(*f*)quinoline	0.71	0.65	0.62	0.58	0.50
7-Azafluoranthene	0.50	0.45	0.41	0.36	0.38
1-Azafluoranthene	0.60	0.54	0.49	0.47	0.46
1-Azacarbazol					
Acenaphtho(1,2*b*)acridine	0.48	0.44	0.42	0.37	0.36
Phenazine			0.70		0.81
Dibenzo(*a,c*)phenazine	0.00	0.00			0.00
11,12-Dimethyldibenz(*a,c*)phenazine					0.00

TABLE II

R_F VALUES OF AZO-HETEROCYCLICS DEVELOPED WITH ORGANIC ACIDS

Paper: Whatman 3MM. Temperature: 18–20°. Eluants: aqueous organic acids.

Substance	*CH_2Cl-COOH* 0.17 *N*	*$CHCl_2$-COOH* 0.17 *N*	*CCl_3-COOH* 0.17 *N*
Acridine	0.73	0.70	0.68
2-Methylacridine	0.61	0.63	0.58
3-Methylacridine	0.62	0.63	0.59
Benz(*a*)acridine	0.21	0.21	C
Benz(*c*)acridine	0.31	0.29	0.23
8,12-Dimethylbenz(*a*)acridine	0.18	0.20	0.00
7,9-Dimethylbenz(*c*)acridine	0.14	0.11	0.00
8,10-Dimethylbenz(*a*)acridine	0.15	0.10	0.00
7,10-Dimethylbenz(*c*)acridine	0.15	0.11	0.00
8,10-Dimethylbenz(*c*)acridine	0.07	0.09	0.00
9,12-Dimethylbenz(*a*)acridine	0.11	0.12	0.00
Dibenz(*a,h*)acridine	0.00	0.02	0.00
Dibenz(*a,j*)acridine	0.03	0.03	0.00
Pyrenoline	0.06	0.03	0.00
Benzo(*c*)cinnoline	0.34	0.53	C
9-(2′-Pyridyl)-anthracene	0.30	0.26	0.24
9-(3′-Pyridyl)-anthracene	0.16	0.15	0.08
9-(4′-Pyridyl)-anthracene	0.13	0.12	0.08
10-(2′-Pyridyl)-1,2-benzanthracene	0.02	0.03	0.00
10-(3′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00
10-(4′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00
14-Phenyldibenz(*a,j*)acridine	0.00	0.00	0.00
7-Phenyldibenz(*c,h*)acridine	0.00	0.00	0.00
Acridone	0.11	0.10	0.11
Phenanthridine	0.66	0.63	0.59
1-Azapyrene	0.38	0.38	C
4-Azapyrene	0.39	0.40	C
Benzo(*h*)quinoline	0.68	0.67	0.63
Benzo(*f*)quinoline	0.61	0.63	0.59
3-Methylbenzo(*f*)quinoline	0.62	0.62	C
7-Azafluoranthene	0.43	0.41	C
1-Azafluoranthene	0.50	0.46	0.00
1-Azacarbazol			C
Acenaphtho(1,2*b*)acridine	0.43	0.40	C
Phenazine	0.38	0.41	0.51
Dibenzo(*a,c*)phenazine	0.00	0.00	0.00
11,12-Dimethyldibenz(*a,c*)phenazine	0.00	0.00	0.00

CCl_3-COOH 0.5 N	COOH | COOH 0.1 N	COOH | COOH 0.5 N	COOH | CH_2 | COOH 0.1 N	COOH | $(CH_2)_2$ | COOH 0.1 N	COOH | CH_2 | COOH 0.5 N	CH_3-COOH 0.17 N (= 1%)
0.74	0.72	0.78	0.68	0.58	0.77	0.54
0.69	0.65	0.73	0.59	0.50	0.69	
0.70	0.64	0.71	0.59	0.47	0.67	
0.31	0.24	0.34	0.20	0.15	0.27	0.12
0.33	0.33	0.42	0.26	0.16	0.36	0.10
0.21	0.20	0.34	0.20	0.19	0.28	0.06
0.00	0.11	0.22	0.10	0.07	0.15	0.07
0.00	0.13	0.22	0.10	0.08	0.17	0.07
0.13	0.13	0.23	0.10	0.07	0.18	0.06
0.12	0.11	0.22	0.10	0.07	0.16	0.07
0.16	0.11	0.25	0.10	0.09	0.18	0.00
0.01	0.03	0.01	0.01	0.08	0.01	0.02
0.00	0.02	0.03	0.02	0.02	0.03	0.01
0.07	0.06	0.10	0.03	0.02	0.07	0.01
0.71	0.48	0.69	0.32	0.30	0.48	0.29
0.00	0.28	0.40	0.26	0.24	0.32	0.18
0.00	0.16	0.28		0.00		0.00
0.00	0.14	0.18	0.13	0.08	0.20	0.06
0.03	0.03	0.05	0.27	0.02	0.05	0.02
0.00	0.01	0.03	0.00			0.00
0.00	0.01	0.02	0.01	0.00	0.02	0.00
0.00	0.01	0.00	0.00	0.00	0.03	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.17	0.13	0.19	0.09	0.10	0.09	0.22
0.59	0.65	0.72	0.64	0.52	0.68	
0.46	0.40	0.52	0.35	0.26	0.47	
0.48	0.40	0.50	0.36	0.27	0.49	
0.72	0.68	0.72	0.65	0.54	0.72	
0.69	0.63	0.68	0.59	0.50	0.67	
0.52	0.63	0.71	0.63	0.52	0.69	
0.00	0.43	0.53	0.38	0.27	0.51	
0.00	0.53	0.64	0.43	0.31	0.60	
	0.46	0.53		0.31		0.29
	0.38	C		0.22		0.21
0.00	0.00	0.00	0.00		0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00

J. Chromatog., 37 (1968) 333–340

TABLE III

R_F VALUES OF AZA-HETEROCYCLICS ON PAPER DEVELOPED WITH ACETATE BUFFERS

Paper: Whatman 3MM. Temperature: 18–20°. Eluants: aqueous acetate buffers.

Substance	*pH*										
	0.48	*0.89*	*1.60*	*2.84*	*3.95*	*4.92*	*5.20*	*5.23*	*5.37*	*5.57*	*5.89*
Acridine	0.67	0.68	0.67	0.66	0.61	0.47	0.40	0.30	0.31	0.25	0.20
2-Methylacridine	0.57	0.60	0.59	0.57	0.51	0.31	0.25				
3-Methylacridine	0.58	0.60	0.58	0.58	0.52	0.30	0.25				
Benz(*a*)acridine	0.13	0.16	0.15	0.14	0.09	0.03	0.01				
Benz(*c*)acridine	0.22	0.25	0.25	C	0.00	0.00	0.00				
8,12-Dimethylbenz(*a*)acridine	0.13	0.15	0.15	0.14	0.09	0.03	0.00				
7,9-Dimethylbenz(*c*)acridine	0.06	0.07	0.05	0.05	0.03	0.00	0.00				
8,10-Dimethylbenz(*a*)acridine	0.07	0.09	0.08	0.06	0.02	0.00	0.00				
7,10-Dimethylbenz(*c*)acridine	0.07	0.09	0.08	0.07	0.03	0.00	0.00				
8,10-Dimethylbenz(*c*)acridine	0.07	0.08	0.09	0.06	0.03	0.00	0.00				
9,12-Dimethylbenz(*a*)acridine	0.06	0.07	0.09	0.19	0.05	0.03	0.02				
Dibenz(*a*,*h*)acridine	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
Dibenz(*a*,*j*)acridine	0.00	0.00	0.00	0.00	00.0	0.00	0.00				
Pyrenoline	0.01	0.02	0.02	0.00	0.00	0.00	0.00				
Benzo(*c*)cinnoline	0.68	0.57	0.28	0.28	0.20	0.19	0.20	0.18	0.20	0.12	0.17
9-(2′-Pyridyl)-anthracene	0.22	0.20	0.24	0.20	0.14	0.08	0.08				
9-(3′-Pyridyl)-anthracene	0.13	0.12	0.07	0.00	0.00	0.00	0.00				
9-(4′-Pyridyl)-anthracene	0.07	0.07	0.10	0.05	0.00	0.00	0.00				

10-(2′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00	00.0	0.00	0.00	0.00				
10-(3′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
10-(4′-Pyridyl)-1,2-benzanthracene	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
14-Phenyldibenz(*a*,*j*)acridine	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
7-Phenyldibenz(*c*,*h*)acridine	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
Acridone	0.10	0.11	0.11	0.10	0.09	0.09	0.08				
Phenanthridine	0.60	0.61	0.65	0.56	0.37	0.12					
1-Azapyrene	0.33	0.35	0.36	0.27	0.10	0.03	0.02				
4-Azapyrene	0.33	0.37	0.35	0.24	0.10	0.03	0.02				
Benzo(*h*)quinoline	0.63	0.64	0.63	0.54	0.34	0.14	0.15				
Benzo(*f*)quinoline	0.56	0.58	0.58	0.56	0.46	0.21	0.17				
3-Methylbenzo(*f*)quinoline	0.55	0.57	0.56	0.59	0.53	0.33	0.29				
7-Azafluoranthene	0.36	0.36	0.36	0.26	0.10	0.03	0.04				
1-Azafluoranthene	0.42	0.44	0.42	0.24	0.09	0.02	0.02				
1-Azacarbazol	0.57	0.57	0.55	0.31	0.12	0.06	0.05				
Acenaphtho(1,2*b*)acridine	0.37	0.38	0.37	0.23	0.11	0.04	0.05				
Phenazine	0.64	0.48	0.28	0.20	0.21	0.19	0.17	0.12	0.12	0.11	0.12
Dibenzo(*a*,*c*)phenazine	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
11,12-Dimethyldibenz(*a*,*c*)phenazine	0.00	0.00	0.00	0.00	0.00	0.00	0.00				

pH 0.48 to pH 5.20: HCl/CH_3COONa buffers.
pH 5.23 to pH 5.89: CH_3COOH/CH_3COONa buffers.

In this note we report on results obtained with some further paper chromatographic systems, as well as with polyamide thin layers.

Paper chromatographic systems using aqueous acids as eluants

Preliminary results indicated that sulphuric acid gave some improved separations of some pairs of low R_F compounds.

Table I reports R_F values for various concentrations of aqueous sulphuric acid. There is a general trend of decreasing R_F values with the increase in the H_2SO_4 concentration, except for benzo-(*c*)-cinnoline.

Table II shows the R_F values for various concentrations of some organic acids. The chloracetic acids, with the exception of monochloracetic acid, precipitated many

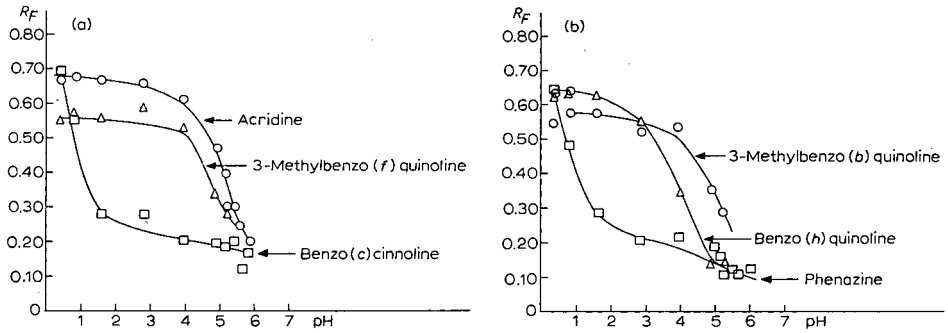

Fig. 1a and b. Some R_F–pH curves for aza-heterocyclics developed with acetate buffers on cellulose paper. Benzoquinolines and acridine have similar curves and can be distinguished clearly from phenazine or benzo(*c*)cinnoline.

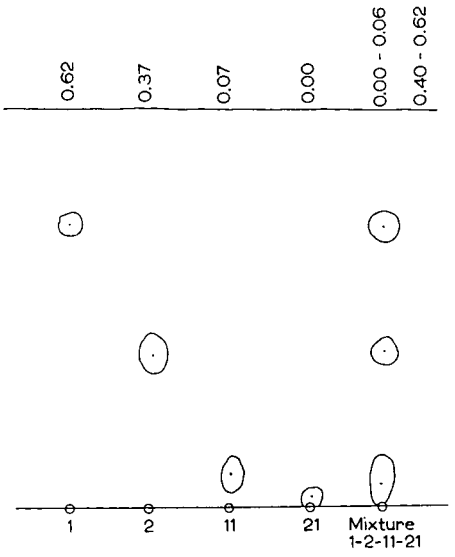

Fig. 2. Polyamide thin layer chromatogram. Solvent: methanol 80%, water 20%. Distance start to solvent front: 69 mm. 1 = Acridine; 2 = benz(*a*)acridine; 11 = dibenz(*a,j*)acridine; 21 = 7-phenyldibenz(*c,h*)acridine. Mixture 1, 2, 11 and 21.

acridines wholly or partially on the point of application and are hence of little use for analytical work. Dicarboxylic acids gave higher R_F values for many acridines than acetic acid; however the sequence remains essentially unchanged.

Paper chromatographic systems with aqueous buffers

There is a marked change of R_F value with all aza-heterocyclics when the pH values are changed by using aqueous acetate buffers as eluants. Table III and Fig. 1 show the possibilities as well as the limitations of pH variation, the latter being that no information can be drawn from low R_F values and that most monoaza-heterocyclics have an inflection point in the same pH range. Comparisons of pK values with paper

TABLE IV

R_F VALUES OF AZA-HETEROCYCLICS ON POLYAMIDE THIN LAYERS

Polyamide thin layers (Cheng Chin Trading Co. Ltd., Taiwan). Temperature: 18–20°.

Substance	*Acetone–water 60%–40%*	*Methanol–water 80%–20%*
Acridine	0.62	0.63
2-Methylacridine	0.58	0.59
3-Methylacridine	0.64	0.62
Benz(*a*)acridine	0.30	0.28
Benz(*c*)acridine	0.23	0.15
8,12-Dimethylbenz(*a*)acridine	0.28	0.16
7,9-Dimethylbenz(*c*)acridine	0.13	0.08
8,10-Dimethylbenz(*a*)acridine	0.11	C
7,10-Dimethylbenz(*c*)acridine	0.16	0.12
8,10-Dimethylbenz(*c*)acridine	0.12	C
9,12-Dimethylbenz(*a*)acridine	0.32	0.46
Dibenz(*a,h*)acridine	0.04	0.00
Dibenz(*a,j*)acridine	0.05	C
Pyrenoline	0.14	0.13
Benzo(*c*)cinnoline	0.73	
9-(2′-Pyridyl)-anthracene	0.37	0.48
9-(3′-Pyridyl)-anthracene	0.41	0.43
9-(4′-Pyridyl)-anthracene	0.40	0.45
10-(2′-Pyridyl)-1,2-benzanthracene	0.18	0.26
10-(3′-Pyridyl)-1,2-benzanthracene	0.21	0.25
10-(4′-Pyridyl)-1,2-benzanthracene	0.09	C
14-Phenyldibenz(*a,j*)acridine	0.00	C
7-Phenyldibenz(*c,h*)acridine	0.00	0.00
Acridone	.41	0.41
Phenanthridine	.66	0.62
1-Azapyrene	C	0.51
4-Azapyrene	C	0.45
Benzo(*h*)quinoline	0.65	0.60
Benzo(*f*)quinoline	0.63	0.60
3-Methylbenzo(*f*)quinoline	0.73	0.63
7-Azafluoranthene	0.51	0.51
1-Azafluoranthene	0.55	0.54
1-Azacarbazol	0.58	0.56
Acenaphtho(1,2*b*)acridine	0.55	0.51
Phenazine	0.72	0.70
Dibenzo(*a,c*)phenazine	0.00	0.00
11,12-Dimethyldibenz(*a,c*)phenazine	0.00	0.00

chromatographic behaviour could not be made because very few pK values were found in the literature.

The pH curves may be useful for deciding whether a certain unknown substance is of the acridine type or not.

Thin layer chromatography on ready polyamide layers

Polyamide layers have been used for many classes of compounds notably for phenols (for a review see HÖRHAMMER *et al.*[4]), however acridines have not been studied to our knowledge. Table IV shows the R_F values obtained on ready-made polyamide layers (Cheng Chin Trading Co. Ltd., Taiwan) with acetone–water and methanol–water as solvents. The order of movement is mainly according to molecular weight and hence the sequences are essentially similar to those on cellulose paper. However very clear and fast separations of several artificial mixtures could be obtained readily (see Fig. 2).

This work was carried out as part of the work for a doctorate thesis by S. CAROLI. Some of the work on sulphuric acid solvents was done by G. ROCH.

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Some critical remarks on the colours of secondary amines on paper with sodium nitroprusside reagent

In paper electrophoretic and chromatographic studies on the occurrence of biogenic amines and basic amino acids in the faeces[1–3], rumen fluid[4] and urine[5] of dairy cows, we used ninhydrin as a colour reagent, though in some cases diazotized sulfanilic acid (Pauly's reagent) was used for the final detection of aromatic compounds. Generally good results had been found with the ninhydrin reagent, but the colours with the secondary amines, dimethylamine and diethylamine, were often faint.

TABLE I

RELATIVE INTENSITY OF BIOGENIC AMINES AND BASIC AMINO ACIDS COLOURED WITH SODIUM NITROPRUSSIDE REAGENT

Compound	*Concentration in μg*	*Paper: Schleicher & Schüll 2043 b*			
		Untreated	*Treated with**		
			A	*B*	*C*
Methylamine	2.8	+	—	—	—
Dimethylamine	1.7	+	—	—	(+)
Ethylamine	2.5	+	—	—	—
Diethylamine	3.6	+	—	—	+
Propylamine	3.1	+	—	—	—
Butylamine	3.8	+	—	—	—
Amylamine	3.8	+	—	—	—
Phenylethylamine	3.1	+	—	—	—
Tyramine	3.9	+	—	(+)	—
Diaminopropane	3.1	+	—	(+)	—
Putrescine	3.3	+	—	+	+
Cadaverine	3.5	+	—	+	+
Spermine	4.0	+	—	((+))	((+))
Spermidine	4.0	+	—	(+)	((+))
Ethanolamine	2.0	+	—	—	(+)
3-Aminopropane	3.0	+	—	—	((+))
4-Aminobutane	4.0	+	—	((+))	((+))
Agmatine	3.4	+	—	(+)	—
Lysine	3.4	+	—	(+)	(+)
Arginine	3.4	+	—	—	(+)
Histidine	2.9	+	—	—	—
Ornithine	2.3	+	—	(+)	—
β-Alanine	2.5	+	—	—	—
γ-Aminobutyric acid	2.5	+	—	—	—
ε-Aminocaproic acid	4.0	+	—	—	—
Taurine	3.0	+	—	—	—
Tryptamine	3.7	+	—	—	—
Histamine	3.9	+	—	—	—
Carnosine	4.0	+	—	—	—
Norephedrine	3.3	+	—	—	—
Kynurenine	3.4	+	—	—	—
Glutamine	4.0	+	—	—	—
Citrulline	2.5	+	—	—	—

* A = Phenol saturated with water; B = *n*-butanol–acetic acid–water (4:1:5); C = ethylene glycol monomethyl ether–propionic acid–water (70:15:15), saturated with sodium citrate. + = Strong; (+) = faint; ((+)) = very faint; — = negative.

In later studies we therefore decided to make use of the colours produced by secondary amines with the sodium nitroprusside reagent recommended by STEIN VON KAMIENSKI[6]. According to this author, this reagent should be highly sensitive and specific for secondary amines.

On spraying an electrochromatogram of a faeces extract, in which dimethylamine and diethylamine were present, however, we found with this reagent a blue spot in the position of spermidine. This unexpected result decided us on a closer study of this colour reagent.

A great number of biogenic amines and basic amino acids were treated on chromatographic paper (Schleicher & Schüll, 2043b) with the sodium nitroprusside reagent. All compounds tested in this way gave a blue-purple spot after 30–60 minutes. The experiment was repeated with chromatographic paper treated beforehand with the solvents used[2,3]. The results of these experiments are given in Table I.

Although STEIN VON KAMIENSKI[6] stated that primary amines give pale red-blue spots with this reagent above 10 μg, we found, however, that this also happened below 10 μg, and that, moreover, the colour was the same as with secondary amines.

Furthermore it appears that the sensitivity depends strongly on the solvent used. Thus we were unable to confirm the great sensitivity and specificity of this reagent.

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Filter paper electrophoresis of purines and pyrimidines: mobility data

In our laboratory, a combination of filter paper electrophoresis and partition chromatography has been applied to the separation and identification of a number of purines, pyrimidines, and nucleosides isolated from urine of normal and leukemic subjects[1]. As a preliminary step, electrophoretic mobilities were obtained for some 94 compounds, and these are reported here.

Experimental

The Durrum Model B paper electrophoresis cell was used with a rack which accommodates eight filter paper strips (Schleicher and Schüll 2043-A, 3 cm × 30.4 cm). Sodium borate buffer, ionic strength of 0.1 at pH 9.2, was used throughout. Aliquots of solutions of the authentic compounds, containing from 2–5 μg, were applied as small spots (2–3 mm) at the center of the paper strips. After the rack of strips was placed in the cell the papers were moistened with buffer to within 1 cm of the initial positions of the applied compounds. The cell was covered, and a potential of 300 V was applied across the strips, generally for 4 h. (Some compounds, especially nucleotides, migrate too rapidly and hence a two-hour period was used for these.) After the filter paper strips had dried at room temperature, the compounds were visualized under ultraviolet light, and the distance of migration, either to the anode or cathode, was measured from the point of origin.

In an attempt to reduce the variation in mobility values obtained with replicate determinations in the same or separate series of runs, the effect of equilibration was studied using the following procedure. A 3 mm hole was punched equidistant from the ends of the paper strips, prior to placing the strips on the rack and positioning it in the cell. The papers were moistened with buffer and allowed to equilibrate in the covered cell for 2 h at room temperature. The standard solutions were applied to individual 3 mm paper discs and allowed to dry. The discs were then inserted with the use of forceps through an aperture in the cover of the Durrum cell and placed in the circular holes previously punched from the paper strips. The aperture was closed, and an additional equilibration period of 30 min was used before the electrophoresis was started, using the same voltage and time as above.

Results and discussion

The mobility data are tabulated in Tables I–IV. In the first three tables, the compounds are arranged according to average mobility, to permit easy recognition of what compounds might overlap and thus be considered in separation and identification procedures. The classification in these three tables was made on the basis of direction of migration (anode or cathode) and the period of time used for electrophoresis (2 or 4 h). In Table IV the compounds, together with average mobilities, are arranged in alphabetical order in order to determine whether a specific compound has been studied and to facilitate location of that compound in the preceding tables.

The fluctuations in mobilities for a given compound are probably inherent in the procedure, with lack of uniformity of filter paper as one variable. The technique with the insertion of the discs to which the standard has been applied permitted

TABLE I

ELECTROPHORETIC DATA FOR PURINES AND PYRIMIDINES AND THEIR DERIVATIVES ARRANGED ACCORDING TO MOBILITIES TOWARD THE ANODE

Compound	*Mobility (cm/4 h)*			*Number of Determinations*
	Average	*Range*		
Xanthosine	11.5	13.1	11.2	3
5-Fluorouracil	11.3	11.5	11.0	3
Xanthine	9.3	9.9	8.3	14
Inosine	8.3	8.8	7.9	4
8-Hydroxypurine	8.0	8.6	7.3	5
1,3-Dimethyluric acid	7.9	8.0	7.8	3
1-Methylxanthine	7.9	8.3	7.3	3
7-Methyluric acid	7.6	7.8	7.4	3
6,8-Dihydroxypurine	7.4	7.8	7.0	6
3-Methyluric acid	7.4	7.7	7.0	4
Uric acid	7.4	7.6	7.4	3
1-Methyluric acid	7.2	7.6	6.9	3
2-Oxypurine	7.1	7.9	6.8	6
Uridine	6.9	7.2	6.3	3
3,7-Dimethyluric acid	6.8	7.2	6.4	3
7-Methylxanthine	6.8	7.2	6.6	4
1,7-Dimethyluric acid	6.6	7.0	6.2	3
5-Fluorodeoxyuridine	6.6	6.9	6.4	3
3-Methylxanthine	6.4	7.0	6.1	5
Guanosine	6.2	6.5	6.1	3
5-Hydroxymethyluridine	6.1	6.4	5.8	5
5-Ribosyluracil	5.7	6.3	5.2	5
1,3-Dimethylxanthine	5.5	5.8	5.3	5
1,7-Dimethylxanthine	5.5	5.8	5.3	5
Thymine ribonucleoside	5.5	5.7	5.3	3
5-Acetylamino-6-amino-3-methyluracil	5.4	5.5	5.4	3
N^2-Dimethylguanosine	5.2	5.6	4.8	22
Inosine-5′-phosphate	5.1	5.7	4.4	3
Hypoxanthine	5.0	5.3	4.7	7
1-Methylinosine	4.8	5.3	4.5	13
4-Amino-5-imidazolecarboxamide ribonucleoside	4.5	4.9	4.2	4
6-Ethylaminopurine	4.4	5.2	4.0	5
Cytidine	4.3	4.6	4.0	3
Adenosine	4.1	4.3	4.0	3
Isoguanine	4.0	4.6	4.0	3
N^6-Methyladenosine	4.0	4.2	3.8	16
1-Methylguanosine	3.7	4.0	3.0	16
2-Amino-8-hydroxypurine	3.2	3.9	2.9	6
7-Methylhypoxanthine	3.1	3.2	3.0	3
8-Hydroxy-7-methylguanine	3.0	3.3	2.7	3
1-Methylhypoxanthine	3.0	3.4	2.8	3
6-Amino-8-hydroxypurine	2.9	3.2	2.6	5
Deoxyinosine	2.9	3.0	2.9	5
9-Methylhypoxanthine	2.6	2.9	2.3	4
Uracil	2.5	2.8	2.2	7
2-Amino-6,8-dihydroxypurine	2.2	2.2	2.2	2
2-Methylhypoxanthine	2.1	2.4	1.9	3
Deoxyguanosine	1.8	1.9	1.7	3
7-Methylguanine	1.8	2.3	1.2	7
3-Methylcytidine	1.8	2.2	1.3	15
Deoxyuridine	1.7	1.8	1.6	3
N^2-Dimethylguanine	1.5	1.8	1.2	3

(*continued on p. 345*)

TABLE I (*continued*)

Compound	*Mobility (cm/4 h)*			*Number of Determinations*
	Average	*Range*		
1-Methyladenosine	1.4	1.7	1.1	14
Guanine	1.3	1.4	1.1	7
5-Hydroxymethyldeoxyuridine	1.3	1.7	1.1	4
N^2-Methylguanine	1.2	1.3	1.0	4
2-Aminopurine	1.1	1.2	0.7	5
6-Methyluracil	0.3	0.7	−0.7	6
Thymidine	0.3	0.6	0.0	5
3-Methyluracil	0.2	0.4	−0.2	5
1-Methyluracil	0.1	0.4	−0.1	5

equilibration of the paper and samples prior to electrophoresis, but with the equilibration times employed, there did not appear to be a significant improvement in the reproducibility.

TABLE II

ELECTROPHORETIC DATA FOR PURINES AND PYRIMIDINES AND THEIR DERIVATIVES ARRANGED ACCORDING TO MOBILITIES TOWARD THE ANODE

Compound	*Mobility (cm/2 h)*			*Number of Determinations*
	Average	*Range*		
6-Succinoaminopurine	8.4	9.0	7.9	4
Orotidine-5′-phosphate	8.1	8.6	7.7	3
Orotidine cyclohexyl ammonium salt	7.3	7.6	7.0	4
Uridine-2′,3′-phosphate	7.3	7.5	6.9	3
Orotic acid	7.2	7.6	6.9	4
Thymidine-5′-phosphate	7.1	7.4	6.9	3
Cytidine-2′,3′-phosphate	6.7	6.9	6.5	3
Guanosine-2′,3′-phosphate	6.5	6.9	6.2	4
Deoxycytidine-5′-phosphate	6.4	7.0	6.2	3
5-Methylorotic acid	6.4	6.7	6.3	3
Adenosine-2′,3′-phosphate	6.3	6.7	5.9	3
Deoxyguanosine-5′-phosphate	6.2	6.4	6.1	3
Deoxyadenosine-5′-phosphate	6.0	6.3	5.8	3

The electrophoretic mobility data included in the tables have been of use in the identification of a variety of purines and pyrimidines found in urine. Because of overlapping mobilities it is often not possible to separate the components of a mixture using electrophoresis for the separations of these compounds in urine. As reported previously[1], we have first used ion-exchange chromatography to obtain a crude fraction. Subsequently filter paper partition chromatography with *n*-butanol, water and concentrated ammonium hydroxide (86:14:1) was employed to provide some separation of the ultraviolet absorbing components, and for the most part move them away from interfering fluorescent substances. Finally, use of electrophoresis in borate buffer provided a second-dimensional resolution, and yielded more readily identifiable spots.

TABLE III

ELECTROPHORETIC DATA FOR PURINES AND PYRIMIDINES AND THEIR DERIVATIVES ARRANGED ACCORDING TO MOBILITIES TOWARD THE CATHODE

Compound	*Mobility (cm/4 h)*			*Number of Determinations*
	Average	*Range*		
3-Methylcytosine	3.4	3.8	3.1	10
Cytosine	2.7	3.1	2.3	7
1,3,7-Trimethylxanthine	2.6	2.8	2.2	4
1,3-Dimethyluracil	2.6	2.8	2.5	3
1-Methyladenine	2.6	3.0	2.1	12
1,3,7,9-Tetramethyluric acid	2.3	2.7	2.1	3
5-Methylcytosine	2.3	2.7	2.0	3
Deoxycytidine	2.3	2.4	2.2	3
4-Amino-5-imidazolecarboxamide	2.1	2.2	1.9	4
7-Methyladenine	1.9	2.1	1.7	4
Deoxyadenosine	1.9	2.0	1.7	4
1,7-Dimethylguanine	1.9	2.2	1.6	5
9-Methyladenine	1.8	2.2	1.5	4
2,6-Diamino-7-methylpurine	1.7	1.8	1.7	3
1-Methylguanine	1.1	1.4	0.8	3
N^6-Methyladenine	0.9	1.2	0.5	3
2-Methyladenine	0.9	1.1	0.7	6
Thymine	0.8	1.0	0.7	7
3,7-Dimethylxanthine	0.5	1.1	0.0	4
Adenine	0.1	0.5	0.1	7

TABLE IV

ALPHABETICAL ARRANGEMENT OF COMPOUNDS, TOGETHER WITH AVERAGE MOBILITY[a]

5-Acetylamino-6-amino-3-methyluracil	5.4	N^2-Dimethylguanosine	5.2
Adenine	0.1[c]	1,3-Dimethyluracil	2.6[c]
Adenosine	4.1	1,3-Dimethyluric acid	7.9
Adenosine-2′,3′-phosphate	6.3[2]	1,7-Dimethyluric acid	6.6
2-Amino-6,8-dihydroxypurine	2.2	3,7-Dimethyluric acid	6.8
2-Amino-8-hydroxypurine	3.2	1,3-Dimethylxanthine	5.5
6-Amino-8-hydroxypurine	2.9	1,7-Dimethylxanthine	5.5
4-Amino-5-imidazolecarboxamide	2.1[c]	3,7-Dimethylxanthine	0.5[c]
4-Amino-5-imidazolecarboxamide ribonucleoside	4.5	6-Ethylaminopurine	4.4
2-Aminopurine	1.1	5-Fluorodeoxyuridine	6.6
Cytidine	4.3	5-Fluorouracil	11.3
Cytidine-2′,3′-phosphate	6.7[2]	Guanine	1.3
Cytosine	2.7[c]	Guanosine	6.2
Deoxyadenosine	1.9[c]	Guanosine-2′,3′-phosphate	6.5[2]
Deoxyadenosine-5′-phosphate	6.0[2]	5-Hydroxymethyldeoxyuridine	1.3
Deoxycytidine	2.3[c]	8-Hydroxy-7-methylguanine	3.0
Deoxycytidine-5′-phosphate	6.4[2]	5-Hydroxymethyluridine	6.1
Deoxyguanosine	1.8	8-Hydroxypurine	8.0
Deoxyguanosine-5′-phosphate	6.2[2]	Hypoxanthine	5.0
Deoxyinosine	2.9	Isoguanine	4.0
Deoxyuridine	1.7	Inosine	8.3
2,6-Diamino-7-methylpurine	1.7[c]	Inosine-5′-phosphate	5.1
6,8-Dihydroxypurine	7.4	1-Methyladenine	2.6[c]
1,7-Dimethylguanine	1.9[c]	2-Methyladenine	0.9[c]
N^2-Dimethylguanine	1.5	7-Methyladenine	1.9[c]
		9-Methyladenine	1.8[c]

(*continued on p. 347*)

TABLE IV (*continued*)

N^6-Methyladenine	0.9[c]	1-Methylxanthine	7.9
1-Methyladenosine	1.4	3-Methylxanthine	6.4
N^6-Methyladenosine	4.0	7-Methylxanthine	6.8
3-Methylcytidine	1.8	Orotidine cyclohexyl ammonium salt	7.3[2]
3-Methylcytosine	3.4[c]	Orotidine-5′-phosphate	8.1[2]
5-Methylcytosine	2.3[c]	Orotic acid	7.2[2]
1-Methylguanine	1.1[c]	2-Oxypurine	7.1
7-Methylguanine	1.8	5-Ribosyluracil	5.7
N^2-Methylguanine	1.2	6-Succinoaminopurine	8.4[2]
1-Methylguanosine	3.7	1,3,7,9-Tetramethyluric acid	2.3[c]
1-Methylhypoxanthine	3.0	Thymidine	0.3
2-Methylhypoxanthine	2.1	Thymidine-5′-phosphate	7.1[2]
7-Methylhypoxanthine	3.1	Thymine	0.8[c]
9-Methylhypoxanthine	2.6	Thymine ribonucleoside	5.5
1-Methylinosine	4.8	1,3,7-Trimethylxanthine	2.6[c]
5-Methylorotic acid	6.4[2]	Uracil	2.5
1-Methyluracil	0.1	Uric acid	7.4
3-Methyluracil	0.2	Uridine	6.9
6-Methyluracil	0.3	Uridine-2′,3′-phosphate	7.3[2]
1-Methyluric acid	7.2	Xanthine	9.3
3-Methyluric acid	7.4	Xanthosine	11.5
7-Methyluric acid	7.6		

[a] The superscript 2 designates mobility toward anode in 2 h; c designates mobility toward cathode in 4 h; all other mobilities are toward the anode in 4 h.

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CHROM. 3645

Zur papierelektrophoretischen Trennung des Psychopharmakons Tofranil®* und einiger seiner Metaboliten**

Mit Hilfe der Papierelektrophorese lassen sich unter erhöhten Spannungen geringere Substanzmengen nachweisen als bei der Papierchromatographie, da es zur Ausbildung sehr schmaler, scharf begrenzter Banden kommt. Die Anwendung der Papierelektrophorese schien uns daher bei Untersuchungen über die Aufnahme des Tofranil (Imipramin) in das Rattengehirn und seinen Metabolismus im Gehirn besonders geeignet, da nur 0.2% des der Ratte applizierten Tofranil in das Gehirn gelangen[1].

Es wurden Versuche durchgeführt mit dem Ziel, das Tofranil und seine 3 wichtigsten Metaboliten Desmethylimipramin (DMI), Didesmethylimipramin (DDMI) und Iminodibenzyl (IDB) voneinander zu trennen.

Trotz zahlreicher Versuche mit den verschiedensten Elektrolyten, mit unterschiedlichen pH-Werten und Spannungen ist es nicht gelungen, eine Trennung der 4 genannten Substanzen unter gleichen Versuchsbedingungen zu erreichen. Zur papierelektrophoretischen Trennung von Tofranil, DMI, DDMI und IDB sind 3 verschiedene Versuchsbedingungen erforderlich (Tabelle 1).

TABELLE I

VERSUCHSBEDINGUNGEN ZUR PAPIERELEKTROPHORETISCHEN TRENNUNG VON TOFRANIL (IMIPRAMIN), DMI, DDMI UND IDB

Die Versuchszeit betrug stets 1 Stunde. Die Banden sind schmal und scharf begrenzt.

Elektrolyt	*Spannung*	*Substanzen*	*Wanderungsstrecke (cm)*	*Getrennt werden*	*Nicht getrennt werden*
1%-ige wässrige Essigsäure	500 V	Imipramin	8.0	DDMI	Imipramin
		DMI	7.8	IDB	DMI
		DDMI	8.8		
		IDB	0		
TRIS–HCl-Puffer, pH: 8.4 1:4 verdünnt	750 V	Imipramin	4.5		
		DMI	6.0	DMI	Imipramin
		DDMI	4.5	IDB	DDMI
		IDB	0		
TRIS–HCl-Puffer, pH: 9.0 1:4 verdünnt	500 V	Imipramin	3.0	Imipramin	DMI
		DMI	6.0	IDB	DDMI
		DDMI	5.6		
		IDB	0		

Als Trägerpapier wurde in allen Fällen Schleicher und Schüll 2043b Mgl verwendet. Die Startpositionen lagen stets 9 cm vom anodischen Ende des Papierstreifens entfernt[2]. Alle Substanzen wanderten zur Kathode hin. Die Bildung Joule'scher Wärme in den Streifen wurde durch Verdünnen der Elektrolyte umgangen[3].

* Geschütztes Warenzeichen der J. R. Geigy, A.G., Basel.

** Mit Unterstützung der Deutschen Forschungsgemeinschaft.

Der Nachweis des Psychopharmakons und seiner Metaboliten erfolgte mit dem H_2SO_4-Reagens nach CRAMMER UND SCOTT[4], das von SCHNEIDER[5,6] modifiziert wurde.

Das Imprägnieren des Trägerpapieres mit Arachidöl, das von HERRMANN[7] für die Papierchromatographie der untersuchten Substanzen empfohlen wurde, erwies sich bei der Papierelektrophorese als ungünstig.

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CHROM. 3662

Bedeutung und zerstörungsfreie Messung der Schichtdicke von Dünnschichtchromatogrammen sowie deren Abstandsmessung am Radiodünnschichtchromatographen

Der Einfluss der Schichtdicke von Dünnschichtchromatogrammen auf die Reproduzierbarkeit der R_F-Werte ist umstritten. Während einerseits Schichtdickenänderungen zwischen 200 and 300 μm keine Einflussnahme zugeschrieben wird[1], zeigen Untersuchungen von PATAKI UND KELEMEN[2] und eigene Versuche, dass auch in diesem Bereich bei einem Vergleich von manuell und mit einem Streichgerät hergestellten Dünnschichtchromatogrammen, erstere infolge einer grösseren Diskontinuität der Schicht 2- bis 4-fach höhere Standardabweichungen (s_{R_F}) der R_F-Werte aufweisen.

Untersuchungen zur Frage der Nachweisempfindlichkeit auf Dünnschichtchromatogrammen lassen den Einfluss von Schichtdickenänderungen auch auf die Erfassungsgrenze qualitativer Farbreaktionen erkennen[3]. Bei Schichtdicken von 90, 175 und 350 μm (Kieselgel G Merck) zeigt z.B. die Antimontrichlorid–Digitoxin-Reaktion mit zunehmender Schichtdicke einen Abfall der Nachweisempfindlichkeit im sichtbaren Bereich von 0.5 auf etwa 2.5 μg und im U.V. von 0.05 auf 0.5 μg, wobei im U.V.-Bereich auch die Möglichkeit einer stärkeren Fluoreszenslöschung in Erwägung gezogen werden muss.

Von wesentlicher Bedeutung erweisen sich Schichtdickenänderungen für die Nachweisgrenze und Reproduzierbarkeit radiometrischer Messungen an Dünnschichtchromatogrammen[4,5]. Dies gilt im besonderen Masse für energiearme β-Strahler. Bei der radiodünnschichtchromatographischen Aktivitätsmessung tritiummarkierter Dünnschichtchromatogramme bedingt ein Anstieg der Schichtdicke von 100 auf 300 μm bei konstanter Ausgangsaktivität einen Abfall der Messeffektivität um 60 %[5].

Auch bei der Verwendung von Streichgeräten können bei gleichbleibender Streichdicke die getrockneten Schichten je nach Korngrösse des Sorptionsmittels[6], Zusammensetzung und Herstellung der Suspension, der Streichgeschwindigkeit und der angewandten Trocknungsbedingungen erhebliche Abweichungen aufweisen[3]. Kontrollmessungen zeigten, dass bei ungünstiger Streichführung sowie Nichteinhaltung der genannten Bedingungen zwischen den Streichchargen, in besonderen Fällen auch zwischen einzelnen Platten derselben, Schichtdickendifferenzen der getrockneten Schicht bis $\pm$ 20 % möglich sind.

Die für die Beurteilung dieser und ähnlicher Fragen gegebenenfalls erforderlichen Schichtdickenmessungen können mittels der nachstehend beschriebenen, praktisch zerstörungsfreien Methode auch im Rahmen von Routineuntersuchungen mit ausreichender Genauigkeit durchgeführt werden. Sie erfolgen als einfache Differenzmessung zwischen Schichtoberfläche und -unterlage (Glasplatte) unter Verwendung einer mit einem spitz ausgezogenen Messfinger versehenen stationären Messuhr. Bei einem Skalenwert von 10 μm beträgt der Gesamtmessbereich 25 mm. Der Wert der Schichtoberfläche wird ohne Beschädigung der Trägerschicht mit Hilfe eines schräg einfallenden kräftigen Lichtstrahles (Mikroskopierleuchte) nach dem Schattenprinzip (Fig. 1) ermittelt, während die Unterlage am nicht beschichteten Plattenrand oder

durch Durchstossen der Schicht ausgemessen wird. Der mittlere Fehler der Messwerte beträgt bei einer Schichtdicke von 200 μm $\pm$ 4.2 % ($n = 10$)*.

Die quantitative Auswertung radiodünnschichtchromatographischer Aktivitätsmessungen tritiummarkierter Dünnschichtchromatogramme erfordert neben einer gleichbleibenden Schichtdicke der Chromatogramme u.a. die Vorlage eines *reproduzierbaren* Minimalabstandes zwischen Zählrohrblende und Chromatogrammober-

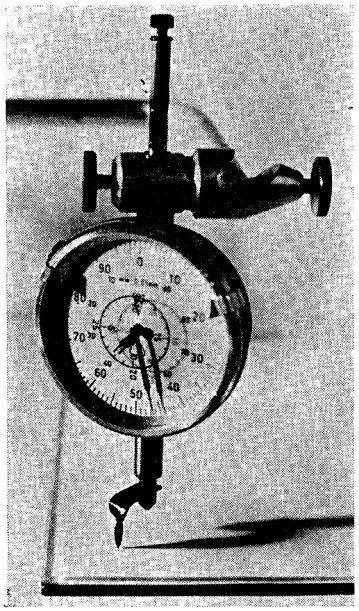

Fig. 1. Schichtdickenmessung durch zerstörungsfreie Abtastung der Chromatogramm-Oberfläche nach dem Schattenprinzip.

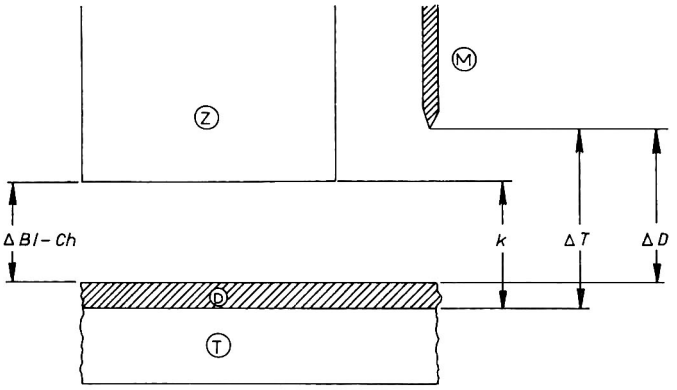

Fig. 2. Messung des Abstandes (Δ_{Bl-Ch}) zwischen Zählrohrblende (Z) und Dünnschichtchromatogramm (D) am Radiodünnschichtchromatographen. M = Messuhr, T = Transportschlitten. (Erklärung im Text.)

* $s = \pm \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$.

fläche[4,5,7,8]. Voraussetzung hierfür ist eine genügend empfindliche und routinemässig durchzuführende Abstands*messung*, bei der eine Beschädigung der Schichtoberfläche aus Kontaminationsgründen unbedingt vermieden werden muss. Bei Verwendung von Radiodünnschichtchromatogrammen mit vom Normalmass abweichenden Glasstärken und Trägerschichtdicken kommt dieser Forderung besondere Bedeutung zu.

Die Abstandsmessung zwischen Zählrohrblende und Chromatogramm ($\Delta_{Bl\text{-}Ch}$) kann in Verbindung mit einer in entsprechender Genauigkeit möglichen Höhenverschiebung des Detektors (stationäre Messskala) durch eine indirekte Differenzmessung nach dem Prinzip der Schichtdickenmessung erfolgen (Fig. 2). Nach einmalig vorgenommener Justierung der Messanordnung durch die Werte k_0 (Wert der Höhenverschiebungsskala des Detektors bei direkter Auflage der Zählrohrblende auf dem Transportschlitten T) und Δ_T (Abstand: stationäre Messuhr–Transportschlitten) ist für jedes neue Chromatogramm lediglich die Ermittlung des mit Δ_D gekennzeichneten Abstandes zwischen Messuhr und Chromatogramm-Oberfläche nach dem Schattenprinzip (Fig. 1) erforderlich. Durch Berechnung von $\Delta_{Bl\text{-}Ch_0}$ (Abstand Zählrohrblende–Chromatogramm-Oberfläche = 0) entsprechend der Beziehung

$$\Delta_{Bl-Ch_0} = k_0 - (\Delta_T - \Delta_D)$$

und Addition des für den jeweiligen Messvorgang gewünschten Abstandswertes können Minimalabstände zwischen Zählrohrblende und Schichtoberfläche bis 50 μm reproduzierbar eingestellt werden. Die relativen Standardabweichungen der Einstellwerte* betragen für $\Delta_{Bl\text{-}Ch}$ 0.5 mm $\pm$ 3.4 % und für 1.0 mm $\pm$ 2.7 % ($n = 10$).

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* Siehe Fussnote auf Seite 351.

CHROM. 3681

Polyamide layer chromatography

XIX. Repeated use of polyamide layers

Polyamide layer chromatography has been used in the analysis of 1-dimethylamino-naphthalene-5-sulfonyl-(DANS)-amino acids[1], dinitrophenyl-(DNP)-amino acids[2], phenylthiohydantoins (PTH) of amino acids[3], lactones[4], nucleobases, nucleosides[5] and nucleotides[6] with satisfactory results. Recently we devised a method of washing used polyamide layers. The washed layers were found as good as new ones even after five repeated uses. This paper describes the method of washing acidic, neutral and basic substances from polyamide layers and shows their reproducibility.

Materials

The polyamide layers were prepared according to WANG *et al.*[7]. 2,4-Dinitrophenyl amino acids, inosine and coumarin were used as samples. These samples were the same quality as was used before (see refs. 2, 4 and 5). All solvents were purified to meet chromatographic requirements.

Methods

The chromatographic method was the same as was used in earlier work[2,4,5]. The chromatograms were washed with two kinds of wash solution; (A) acetone–ammonia water (29 % NH_3) (9:1, v/v) and (B) acetone–90 % formic acid (9:1, v/v). After soaking the used layers in either of the two wash solutions for 6 h, the layers were washed several times with purified methanol, then hung up and air dried. Afterwards, hot air (about 70°) was blown over the air dried layers for a few seconds only to make sure that they were completely dry; longer blowing caused damage to the polyamide layers.

Results and discussion

.ble I shows the R_F values obtained for DNP-alanine (acidic), inosine (basic) and coumarin (neutral) after each washing. The R_F values on layers used five times repeatedly were almost the same. The developing times were also the same after each

TABLE I

THE R_F VALUES OF REPEATEDLY USED POLYAMIDE LAYERS

Samples	*R_F value for repeat no.*					*Solvent**	*Development time (min)*
	1	*2*	*3*	*4*	*5*		
DNP-alanine	0.50	0.50	0.50	0.50	0.50	I	60 ± 3
Inosine	0.54	0.55	0.55	0.55	0.55	II	95 ± 3
Coumarin	0.45	0.45	0.45	0.45	0.45	III	65 ± 3

* Solvent system: (I) Benzene–glacial acetic acid (4:1, v/v); (II) acetone–glacial acetic acid (9:1, v/v); (III) water–90 % formic acid (9:1, v/v).

washing. This meant that the polyamide layer did not change its sorption characteristics after several developing and washing processes.

The wash solution (A) was good for acidic substances. The solution (B) was excellent for basic and neutral types of samples. The dipping time in wash solution (A) should not exceed more than 6 h, otherwise the polyamide layers would peel off. In wash solution (B), the sheets were not harmed even when left for over 3 days.

It was found that the layers should be dipped into the wash solution immediately after development and location of the spots. The samples will be sorbed irreversibly after long standing (for example, overnight).

It is clear from these results that polyamide layers could be used repeatedly. In theory, they can be used an infinite number of times, but mechanical damage caused by handling and decomposition of polyamide resin by developing solvents will restrict it to somewhere around ten times. We have used washed layers in quantitative analysis of DNP-amino acids successfully[8].

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CHROM. 3703

Simple preparative thin-layer chromatography

Numerous attempts have been made to exploit the advantages of thin-layer chromatography for preparative work. Such methods are inconvenient, quite apart from the increase in scale required. Thus to elute the components of mixtures directly from chromatograms needs special apparatus and procedures[1,2] and to recover components by extraction involves subsequent removal of fine adsorbent particles. For best results prior concentration "on the layer" by additional chromatographic steps[3,4] is needed. Consequently most workers still use thin-layer chromatography only for analysis and for preparative work move to classical column chromatography[5]

or to the newer "dry column" method[6]. In the two methods described here these difficulties are avoided, thus opening the way to more widespread use of thin-layer chromatography as a preparative tool. Method No. 1 is extremely simple and method No. 2 though slightly more complex has the advantage of being able to cope with about ten times the load per run.

Method No. 1 needs only a chromatography tank with an upper solvent reservoir (as used for descending paper or thin-layer chromatography) and pre-coated thin-layer sheets with flexible plastic backings (alumina, cellulose and silica adsorbent layers may all be used) such as the "Polygram" and "Chromagram" ranges produced by Macherey Nagel and Eastman, respectively. The chromatogram sheet is prepared for use as follows. Edge effects during development are minimised by scraping off 0.5 cm strips of adsorbent from the vertical edges of the sheet (the completed chromatogram sheet is shown in Fig. 1). To ensure that solvent drops will readily fall from the sheet during development its bottom edge is trimmed slightly obliquely leaving at the lowest corner a very sharply pointed 1.0 cm long "tail". Resolution of closely spaced components is aided if adsorbent is scraped off to form a straight lower adsorbent edge. A right angled fold is now made parallel to and 3 cm from the upper edge of the sheet. The adsorbent layer should be on the inside of the fold to minimise peeling of the adsorbent. A further right angled fold is made parallel to and some 1.5 cm down the sheet from the initial fold. These two folds constitute a "hook" by which the sheet may be hung over the lip of the solvent trough. The developing solvent is chosen with the aid of test strips and direct transfer to the preparative system is possible. Very volatile solvents should be avoided to minimise the risk of the chromatogram drying out. Resolution is often improved by multisolvent development with the mobile components being eluted first by non-polar solvents. The chromatogram is run as follows. The sample is applied as a streak terminating some 1.0–2.0 cm from the edges of the sheet to limit edge effects. The loaded sheet is hooked over the edge of the empty solvent trough and a wire clip placed over the "hook" to prevent it opening

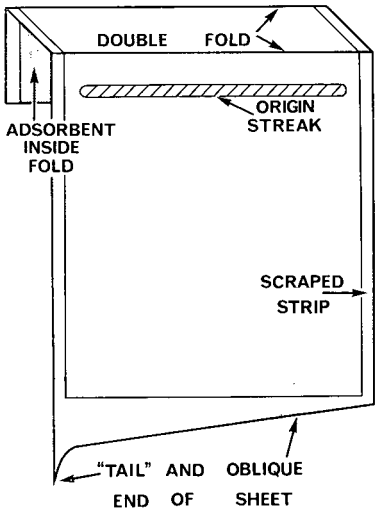

Fig. 1. Completed chromatogram sheet for simple preparative thin layer chromatography using method No. 1.

out during development. Development is started by filling the solvent trough through a previously stoppered port in the tank lid. Separate fractions are collected by moving the sheet along the trough in such a manner that the "tail" delivers successive fractions into successive collecting tubes standing on the floor of the tank. The development of colourless fractions may be followed by using adsorbents containing fluorescent indicators and an ultra-violet lamp arranged to shine through a port in the tank lid. In this case ultra-violet adsorbing aromatic solvents such as toluene cannot be used. A paper wick on the inside of the "hook" will increase the development rate though care must be taken that excessive siphoning and thus irregular flow does not occur.

Method No. 2 at the price of a slight increase in complexity circumvents the major disadvantages of method No. 1, that since the adsorbent layers on pre-coated sheets are only some 0.01 cm thick the useful load is low. Method No. 2 uses 0.1 cm thick layers of any of the standard adsorbents on glass plates, prepared in the usual manner[5] or purchased ready coated, *e.g.* from Analtech, Merck or Schleicher and Schuell. The plates are developed as in method No. 1 by descending continuous elution, wick fed with solvent from the upper trough. The plates stand in narrow glass troughs from which eluted fractions may be pipetted or pumped out. The troughs are flushed out with solvent between fractions to limit mixing. All other relevant details are as described for method No. 1.

These methods can be varied in several useful ways. Rapid measurement of ultra-violet and infra-red spectra is facilitated by collecting fractions directly in cuvettes or on potassium bromide. With multicomponent systems requiring two-dimensional development to obtain good resolution, *e.g.* protein hydrolysates, the chromatogram may be divided into strips after the first development run and the strips turned through 90° and treated as above in order to obtain the individual components.

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The detection of the methylenedioxy group on thin-layer chromatographic plates

A number of natural products contain a methylenedioxy group[1–4] and thus a rapid method for the specific detection of this moiety on thin-layer chromatographic (TLC) plates would be of value both in structural studies and in the identification of these materials. This group has been detected by colour reactions resulting from the treatment of dilute alcoholic solutions with gallic and sulphuric acids[5–7], tannic and sulphuric acids[6] and phloroglucinol and sulphuric acid[8]. The colours, which are apparently due to the reaction of the polyhydroxyphenol with the formaldehyde released upon hydrolysis of the methylenedioxy group[7], vary in shade and intensity and can often lead to ambiguous results.

In contrast to the above reagents, chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulphonic acid) in the presence of sulphuric acid is specific for formaldehyde[9], with which it gives a characteristic purple colour. This reagent has been adapted for the quantitative assay of formaldehyde[10–12] and for the analysis of a variety of methylenedioxy derivatives[13–17]. The chromatography of methylenedioxyphenyl compounds has been the subject of a recent review[18]. This paper describes two modifications of the reagent for its use with TLC for the rapid detection and tentative identification of compounds containing the methylenedioxy group.

Experimental

Test solutions contained 1 mg per 1 ml and were spotted with lambda micropipettes. Chromotropic acid (supplied as the disodium salt) and sulphuric acid were reagent grade. Chromatoplates were prepared at a thickness of 250 μ using MN Kieselgel G-HR. Self-indicating plates were prepared by adding silica gel (25 g) to a solution of sodium chromotropate (1 g) in water (49.5 ml) containing conc. sulphuric acid (0.5 ml). This quantity was sufficient for five 20 × 20 cm plates. All plates were activated for 1 h at 90° after which they were stored in the dark over a silica gel desiccant. The chromotropic acid spray was prepared weekly by slowly adding conc. sulphuric acid (15 ml) to a solution of the sodium salt (1 g) in water (15 ml). Colour development for both sprayed and self-indicating plates was achieved by heating for 15–30 min at 110–120°. The chromatographic solvents used were benzene–petroleum ether (1:1), benzene–1 % methanol and benzene–2 % acetone.

Results and discussion

The detection of the methylenedioxy group with chromotropic acid is based upon the release of formaldehyde and thus a mineral acid must be present in order to effect hydrolysis. Use of the spray reagent (cf. refs. 16 and 17) was found to produce the expected purple colour after heating. Clearly defined spots on a nearly colourless background were obtained when dilute solutions (5–10 γ of applied material) were used; concentrated applications led to colour diffusion together with a purple background. Minimum detectable amounts were in the order of 0.5 γ for safrole, isosafrole, dihydrosafrole, piperonal, piperonol, piperonylic acid, piperonyl acetate, piperonyl isobutyrate and piperine. All compounds were detected as distinct purple spots at this level.

In order to apply direct densitometry to the analysis of several methylenedioxy derivatives of importance in the food industry, the use of self-indicating impregnated plates was investigated. These plates, which contain an even distribution of the chromogenic reagent necessary for densitometric measurements, required only heating for colour development. Although the piperonyl esters were left intact during chromatography in benzene–petroleum ether (1:1), the use of self-indicating plates is necessarily limited to acid-stable compounds. Minimum detection limits (1–2 γ) and general plate appearance were similar to those obtained using the spray mode of detection. However, the latter method is more generally applicable and is to be preferred for routine analysis.

Several other types of compounds are also capable of releasing formaldehyde upon hydrolysis. Hexamethylenetetramine and *sym*-trithiane contain $=N-CH_2-N=$ and $-S-CH_2-S-$ groups, respectively, and thus yield the expected purple spots with the above detection systems. These materials can be distinguished from methylenedioxy derivatives by elemental analysis.

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CHROM. 3638

Improved solvent systems for thin layer chromatography of estrogens*

There are many systems for separation of steroids and other compounds by thin layer chromatography (TLC). Many systems[1–3] were developed by following the separation of pure reference compounds. Consequently, some systems are found to be inadequate when applied to extracts of biological materials. The present paper describes solvent systems for TLC developed for the separation of estrogens in extracts of urine. Unknown substances, which were not separated in "conventional" systems, were separated from the estrogens in the systems containing isopropyl ether.

Experimental

Reagents. Solvents were all of reagent grade and redistilled before use. Ethanol (Publicker Industries) was used as obtained from the supplier. Benzene was washed with dilute sulfuric acid, bicarbonate, and water before distillation. Isopropyl ether was stored over ferrous sulfate and distilled prior to use.

Steroids. The steroids were used as obtained from the supplier. Estrone-16 was obtained through the courtesy of Dr. M. N. HUFFMAN, Creighton University, Omaha, Nebr. The 2-methoxy estrogens were kindly supplied by Dr. JACK FISHMAN, Montefiore Hospital, New York, N. Y. The other estrogens were obtained from commercial sources.

Solvent systems. 1. 7 % ethanol in isopropyl ether; 2. 20 % acetone in isopropyl ether; 3. 10 % ethanol in benzene; 4. Ethyl acetate.

These mixtures were changed in the tanks each day.

Methods. Thin layer plates (obtained from Analtech, Wilmington, Delaware) coated with Silica Gel G (250 microns thickness) were used without prior preparation. Steroid solutions made up in *tert.*-butanol (1 mg/ml) were spotted on the plates using Hamilton microsyringes. Plates, 20 × 20 cm size, and spots of 10 μg steroid were used. These were developed at room temperature until the solvent front had reached 0.5 cm from the top of the plate. The time averaged 40 min. After drying at room temperature, estrogen zones were visualized by spraying with the ferric chloride–potassium ferricyanide reagent[4]. Measurements were made from the center of the spots. The R_F values were calculated in the usual manner.

Results and discussion

Each of the estrogens as listed in Table I was run singly and together with other members of the series. The average value for 4 determinations of the R_F for each of these steroids is given in Table I. For illustrative purposes, an extract of normal female urine was also subjected to separation in each of these systems.

In contemporary TLC systems, the estrone zone of extracts as prepared in these laboratories contains a substance which has the retention time of estradiol-17β on gas chromatography. Likewise, the estradiol-17β zone contains unknown substances. It appeared appropriate to investigate other systems. Thus, the described systems

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TABLE I

R_F (× 100) VALUES FOR ESTROGENS IN DIFFERENT SOLVENTS FOR THIN LAYER CHROMATOGRAPHY
Each figure represents the average of 4 determinations.

Estrogen	*7 % Ethanol in isopropyl ether*	*20 % Acetone in isopropyl ether*	*Ethyl acetate*	*10 % Ethanol in benzene*[2,3]
Substance X	71	67	91	57
Estrone	66	58	93	57
Estrone-16*	64	56	95	56
16-Ketoestrone	65	45	96	51
2-Methoxyestrone	58	52	93	64
Estradiol-17β	51	45	78	40
Estradiol-17α	53	47	70	42
16-Ketoestradiol-17β	42	39	66	55
Estriol	11	7	18	15
Epiestriol	29	23	40	23
2-Methoxyestriol	9	6	16	17

* 3-Hydroxyestra-1,3,5(10)-triene-16-one.

containing isopropyl ether evolved. As seen in Table I, the substance denoted as X migrated with estrone in the systems not containing isopropyl ether. Other materials also separated from the estradiol-17β zone in the systems containing isopropyl ether. For this reason, preliminary purification of the urinary extraction prior to gas chromatography using these systems is carried out in this laboratory.

It will be noted that a greater separation of estriol and epiestriol was obtained in the systems containing isopropyl ether. The system 10 % ethanol in benzene gave the least separation. Different percentages of ethanol in benzene were also investigated, but no separation of X from estrone was obtained. The widest range of separation among substance X, estrone, estradiol-17β and estriol was found in the system 20 % acetone in isopropyl ether. At present, the identity of substance X and other substances separated by the newer systems is unknown.

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Detection of steroids in thin-layer chromatography

The anisaldehyde spray reagent (0.5 ml anisaldehyde, 1 ml conc. sulfuric acid, 50 ml glacial acetic acid) which we have used for detection of bile acids on thin-layer chromatograms[1] gives a variety of colors, depending on the structure of the particular bile acid. This color variability has been useful in assigning structures to unidentified bile acids[2]. We have extended the use of this reagent to detection of a number of other steroids. The results obtained are the basis of this communication.

The steroids (5–10 μg) were spotted on Silica Gel G plates and the chromatogram developed with isooctane–isopropyl ether–acetic acid (2:1:1). The plates were sprayed with the anisaldehyde reagent and kept at 125° for 10 min. The color development was carried out in this manner in order to test the reagent under experimental conditions. Subsequently, groups of similar steroids were also chromato-

TABLE I

COLORS OBTAINED WITH VARIOUS STEROIDS USING ANISALDEHYDE SPRAY REAGENTS

Compound	*Color*	R_F *value*	
		Isooctane–isopropyl ether–acetic acid (2:1:1)	*Benzene–ethyl acetate (4:3)*
Sterols			
Cholesterol	Purple	0.58	0.69
Coprostanol	Purple	0.63	
Sitosterol	Purple	0.56	
Ergosterol	Brown	0.56	0.60
Lanosterol	Purple	0.60	
Desmosterol	Purple	0.53	
Zymosterol	Brown	0.44	
Other steroids			
Δ^4-Cholesten-3-one	Orange	0.56	0.73
3,5-Cholestadiene	Brown	0.90	
7β-Hydroxy-Δ^4-cholesten-3-one	Orange	0.43	0.49
3β,5α,6β-Trihydroxycholestane	Brown	0.22	0.09
Cholesteryl chloride	Purple	0.83	0.80
Cholesteryl laurate	Purple	0.85	
Cholesteryl methyl ether	Purple	0.75	
Androgens			
Androsterone	Purple	0.30	0.50
Epiandrosterone	Purple	0.30	0.50
Testosterone	Brown	0.20	0.41
Estrogens, progestins			
Progesterone	Orange	0.23	0.60
Δ^5-Pregnen-3β-ol-20-one	Purple	0.33	0.50
5α-Pregnane-3β,20β-diol	Purple	0.31	0.43
Estrone	Brown	0.30	0.63
Estriol	Dark purple	0.10	0.12

graphed in benzene–ethyl acetate (4:3) in an attempt to further differentiate specific compounds.

The results are presented in Table I. Among the sterols only zymosterol and ergosterol give colors other than purple. The brown color observed with 3,5-cholestadiene suggests that a conjugated double bond system is responsible for the shade of color obtained. The color obtained with zymosterol cannot be explained on this basis, however, in view of the purple obtained with lanosterol which has the same double bond system, although the 4,4′-methyl groups in the latter compound may exert an effect. The orange color observed with cholestenone and 7β-hydroxycholestenone is similar to the colors obtained with keto bile acids[1]. The brown color obtained with 3β,5α,6β-trihydroxycholestane is probably due to an unsaturated compound obtained as a result of dehydration caused by the spray reagent.

The brown color obtained with testosterone differentiates it from androsterone. The brown color obtained with testosterone and that obtained with estrone indicate that, in the absence of a side chain, the presence of a keto group is insufficient to give the orange shade observed with cholestenone and keto bile acids. This suggestion is given credence by the orange color seen with progesterone.

Our results indicate the further usefulness of the anisaldehyde spray reagent in detection and possibly differentiation of steroids in thin-layer chromatography.

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Resolution of some pairs of closely related steroids by thin-layer chromatography

Work at present in progress in this laboratory has made necessary the development of TLC methods for the resolution of a number of pairs of closely related steroids

TABLE I

SEPARATION OF PAIRS OF CLOSELY RELATED STEROIDS ON STARCH-BOUND KIESELGEL N-HR/UV 254 (MACHEREY, NAGEL)

Steroid pairs[a]	*No. of developments*	*Distance*[b] *moved (cm)*	*Separation factor*
(*a*) *Separations with cyclohexene–cyclohexanone (90:10)*			
5α-Pregnane-3,20-dione	2	7.30	1.21
5β-Pregnane-3,20-dione		6.05	
5α-Androstane-3,17-dione	2	5.40	1.23
5β-Androstane-3,17-dione		4.40	
3β-Hydroxy-5α-pregnan-20-one	2	2.95	1.31
3α-Hydroxy-5β-pregnan-20-one		2.25	
3β-Hydroxy-5α-androstan-17-one	3	5.80	1.49
3α-Hydroxy-5β-androstan-17-one		3.90	
(*b*) *Separations with cyclohexene–cyclohexanone (80:20)*			
3β-Hydroxy-5α-androstan-17-one	1	6.50	1.18
3α-Hydroxy-5β-androstan-17-one		5.50	
3β,17β-Dihydroxy-5α-androstane	2	3.35	1.43
3α,17β-Dihydroxy-5β-androstane		2.35	
3β,20β-Dihydroxy-5α-pregnane	2	4.40	1.33
3α-20β-Dihydroxy-5β-pregnane		3.30	
3β,20α-Dihydroxy-5α-pregnane	2	3.00	1.36
3α,20α-Dihydroxy-5β-pregnane		2.20	
3β,17α-Dihydroxy-5α-pregnan-20-one	2	3.85	1.48
3α,17α-Dihydroxy-5β-pregnan-20-one		2.60	
3β,17α,20β-Trihydroxy-5α-pregnane	3	2.50	1.47
3α,17α,20β-Trihydroxy-5β-pregnane		1.70	
3β,17α,20α-Trihydroxy-5α-pregnane	3	1.80	1.71
3α,17α,20α-Trihydroxy-5β-pregnane		1.05	

[a] Each pair was run as a mixture.

[b] Average of measurements on at least two chromatograms and quoted to nearest 0.5 mm. Solvents were freshly distilled. Plates were prepared by a method described earlier[2] and run in tanks lined with Whatman No. 3 papers. To avoid interference with detecting agents, last traces of cyclohexanone were removed from the plates by lightly spraying with water and drying in warm air. Many steroids were faintly visible while the plates remained damp[3]. The following detecting agents were employed: (1) 0.1% 2,4-dinitrophenylhydrazine in aqueous 2 *N* hydrochloric acid; (2) 10% phosphomolybdic acid in ethanol; (3) 0.002% aqueous rhodamine B, with examination under U.V. light of maximum output 355 mμ or, less satisfactorily, 254 mμ; (4) sulphuric acid–water (1:1, w/v)[5]; this reagent produced some background darkening.

for example, 5α- and 5β-androstane-3,17-dione, 5α- and 5β-pregnane-3,20-dione and 3α-hydroxy-5β- and 3β-hydroxy-5α-pregnan-20-one[1].

Preliminary experiments established that many of the pairs of closely related steroids could be resolved by repeated development on thin-layer plates in solvents of low polarity, for example, cyclohexane with up to 10% v/v of cyclohexanone, acetone or ethyl acetate. Solvents whose polarities had been increased by increasing the proportions of the more polar constituents showed reduced resolving power and were less satisfactory. Where solvents of increased polarity were required it was found advantageous to substitute cyclohexene for cyclohexane. As shown in Table I, solvent mixtures of cyclohexene and cyclohexanone have been used to resolve a number of pairs of closely related steroids of interest in this laboratory. Mixtures of cyclohexene with acetone or ethyl acetate have also been used. Although the disadvantages which accompany use of the high boiling cyclohexanone are thereby avoided, the resolving power of these solvents with some steroid mixtures was inferior to that of the cyclohexene–cyclohexanone systems.

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CHROM. 3702

Un nouveau mode de détection, sur couches minces, des amides substituées

Les amides aliphatiques substituées peuvent être détectées par pulvérisation, soit de sulfate acide d'ammoniaque (suivie de chauffage), soit d'une solution d'iode, soit de techniques plus particulières dans le cas spécial des urées[1].

La méthode que nous proposons consiste dans la pulvérisation successive d'une solution de dinitrophénylhydrazine (DNPH) et d'une solution d'iode.

L'action préalable du DNPH augmente très nettement les limites de détection de l'iode utilisé seul (cf. Tableau I) et, de plus, rend ce processus spécifique. En effet, sur le fond jaune obtenu par la pulvérisation du DNPH l'iode ne met pratiquement

plus en évidence que les amides substituées. Les colorations obtenues, pour de nombreux lipides, par l'utilisation de l'iode seul, sont masquées.

Partie expérimentale

Le travail est effectué en chromatographie ascendante sur plaques Merck préparées D.C.F. 254, épaisseur de silica gel 250 μ.

L'agent de développement est un mélange en volume de 40 parts de cyclohexane, 40 parts d'isopropanol et 20 parts d'acétate d'éthyle.

Dans ce milieu, les valeurs R_F des amides examinées varient pour la plupart de 0.4 à 0.7.

La solution de DNPH dans l'éthanol contient 0.1 g de 2,4-dinitrophénylhydrazine p.a. de Fluka et 10 ml d'HCl concentré (dté 1.19) p.a. de UCB pour un volume de 100 ml.

La solution d'iode contient 1 g d'iode bisublimé de UCB pour 100 ml de méthanol.

TABLEAU I

LIMITES DE DÉTECTION COMPARÉES SUR COUCHES MINCES DÉVELOPPÉES

Amides substituées	*Détection par I_2 seul (γ)*	*Détection par DNPH puis I_2 (γ)*
N-Diéthylacétamide	4	2
N-Dibutylacétamide	4	0.5
N-Dibutylpropionamide	5	1
N-Dibutylbutyramide	5	0.6
N,N'-Tétrabutylsuccinamide	4	1
N-Dibutylundécanamide[a]	4	2
N-Dibutylbenzamide	6	1
N-Méthylformamide	80	40
N-Méthylacétamide	30	20
N-Butylacétamide	20	10
N-Butylpropionamide	8	2
N-Butylbutyramide	6	2
N,N'-Dibutylsuccinamide	8	3
N-Butylundécanamide	4	1
N-Butylbenzamide	6	1

[a] Dans le cas de l'utilisation du sulfate acide d'ammoniaque, cette limite est de 6 γ pour la N-dibutylundécanamide.

Le mode d'application est le suivant: après avoir pulvérisé la solution de DNPH on chauffe la couche mince à 110° pendant 2 min; après refroidissement, on pulvérise la solution d'iode. La position des amides substituées est révélée en quelques secondes par l'apparition de taches brun foncé sur fond jaune pâle.

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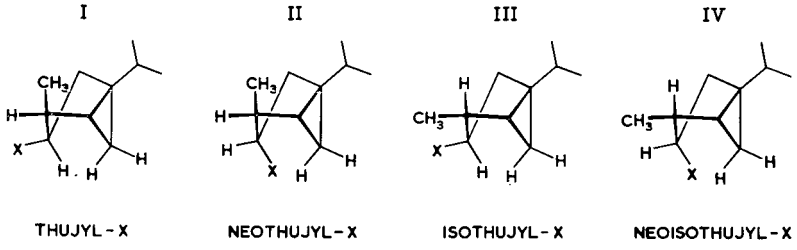
CHROM. 3691

Thin-layer chromatography of thujyl compounds

Column and thin-layer chromatography of certain epimeric compounds, *e.g.* menthylamines[1,2], bornylamines[2], menthols[3], and cyclohexylamines[4], have shown that the isomers with relatively shielded functional groups are eluted more readily by weakly polar solvents. The phenomenon is suggested to be general and to be useful for the rapid assignment of configurations or for indicating preferred conformations for compounds of known configuration[4].

We here report the first systematic thin-layer chromatographic studies of thujyl compounds which enable the generality of these ideas to be extended to a bicyclic system. The thujones, thujols and thujylamines studied are of known configuration, I to IV[5,6], and have been shown by NMR spectroscopy to exist predominantly in *quasi*-boat conformations[5–8].



Experimental

Pure specimens of the thujyl compounds were available[6]. Thin-layer plates (0.25 mm) were prepared with a Shandon apparatus using a slurry of silica gel (Merck G-grade, 30 g) and water (60 g), allowed to set for 5 min and then heated at 110° for 10 min while placed horizontally and for 60 min whilst vertical, before being stored in a desiccator.

Substrates (0.1 μl, 10 % solution in hexane) were applied using a microsyringe and plates were developed with purified solvents (dried over molecular sieves) until the solvent front had risen 10 cm above the base-line. After removal of solvent, the plates were sprayed with a saturated solution of phosphomolybdic acid (analytical reagent grade) in ethanol and then heated at 110° for a few minutes to develop blue spots on a yellow background. Vanillin in concentrated sulphuric acid (2 %, v/v) also gave satisfactory traces, but diphenylpicrylhydrazyl[9] as spray did not give good results.

The R_F values for the best solvent systems are given in Tables I and II.

Discussion

Good analytical separations were achieved. The only previous study in this field, that of thujols using only the conditions A of Table I, gave a similar elution order[10].

Increase of the polarity or hydrogen-bonding ability of the solvent in the sequences A to D and E to F of Table I gave no complete reversal of the order of

TABLE I

THIN-LAYER CHROMATOGRAPHIC DATA FOR THUJOLS

Solvents: A = benzene; B = benzene–ethyl acetate (95:5); C = benzene–ethyl acetate (70:30); D = ethyl acetate; E = benzene–methanol (95:5); F = benzene–methanol (75:25); G = methanol; H = methanol; I = benzene. Conditions: (A–G) Plate activated in conventional manner (see Experimental section). (H,I) Plate activated 100°/0.5 mm for 3 h.

Compound	R_F *values*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
(+)-Neoisothujol	0.27	0.38	0.70	0.80	0.50	0.67	0.74	0.76	0.14
(−)-Neothujol	0.20	0.31	0.62	0.80	0.44	0.66	0.77	0.80	0.09
(−)-Thujol	0.15	0.25	0.58	0.80	0.40	0.66	0.74	0.80	0.06
(+)-Isothujol	0.12	0.20	0.55	0.80	0.36	0.68	0.74	0.82	0.00

TABLE II

THIN-LAYER CHROMATOGRAPHIC DATA FOR THUJYLAMINES

Solvents: A = acetone–40–60° petroleum ether–880 aq. ammonia (50:25:1); B = acetone–40–60° petroleum ether–880 aq. ammonia (25:50:1); C = acetone–40–60° petroleum ether (25:50), saturated with ammonia gas; D = acetone–40–60° petroleum ether (50:25), saturated with ammonia gas.

Compound	R_F *values*			
	A	*B*	*C*	*D*
Amine from (+)-neoisothujol	0.79	0.88	0.91	0.73
Amine from (−)-neothujol	0.73	0.81	0.84	0.62
Amine from (+)-isothujol	0.63	0.71	0.66	0.45
Amine from (−)-thujol	0.55	0.65	0.56	0.33

elution of the thujols; but increased activation of the plates, conditions H and I, did allow such a change. Of the menthols, those isomers with low hydrogen-bonding ability due to relatively shielded hydroxyl groups migrated fastest in non-hydrogen-bonding solvents, whereas in eluting media of considerable hydrogen-bonding capacity the order was reversed, the isomers with less shielded hydroxyls now being favourably partitioned to the solvent[3]. Our results clearly indicate the hydrogen-bonding abilities to be

(+)-Isothujol > (−)-Thujol > (−)-Neothujol > (+)-Neoisothujol

Except for a reversal of the former pair, this order would have been predicted from the conformations I and IV assuming that shielding by the methylene bridge is the major influence on hydrogen-bonding capacity.

Resolution of (−)-thujone and (+)-isothujone (derived from (−)-thujol and (+)-isothujol respectively by oxidation) could only be achieved with either toluene or dichloromethane as solvent when the R_F values were 0.28 and 0.33 (toluene) and 0.55 and 0.58 (dichloromethane). No polar component could be found to reverse the order, but our data are consistent with isothujone existing in a *quasi*-boat analogous

to III or IV and being less bonded to the stationary phase than its epimer in which the *quasi*-axial methyl group provides less shielding of the carbonyl function.

As with the thujones, a solvent pair could not be found that reversed the order of elution of the thujylamines from that of conditions A to D, Table II. However, under the last conditions, (—)-neothujol, (+)neoisothujol, (+)-isothujol, and (+)-sabinene (a terpene hydrocarbon) ran with R_F 0.78, 0.78, 0.74 and 0.80, which proves the conditions to be of the weakly hydrogen-bonding type in which the most sterically-hindered amine ran fastest. The observed order of hydrogen-bonding ability is thus

Thujylamine > Isothujylamine > Neothujylamine > Neoisothujylamine

which is expected from considerations of conformations I to IV, and does not show the reversal of the first pair that is found for the thujols. For the latter the order as determined by shielding by the methyl group is apparently easily reversed by small changes in conditions that are insufficient to alter the broader division caused by shielding by the methylene bridge.

In conclusion, the R_F values of these thujyl compounds can be rationalised on the correlation between configurations, conformations, and R_F values that had been previously proposed, and the results lend confidence to the extension of these ideas to other mono- and bicyclic compounds.

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CHROM. 3641

Use of hydriodic acid in the detection of pesticides after thin-layer chromatography

The use of thin-layer chromatography in the separation of pesticide residues has attracted much attention and many chromogenic reagents have been used in their detection. FINOCCHIARO AND BENSON[1] have extensively studied reagents applicable to the detection of carbamates and ureas, and ABBOTT *et al.*[2] describe several reagents not studied by the former authors. Chromogenic reagents used for the detection of organophosphorus pesticides have been recently reviewed[3–5].

Many of the chromogenic reagents described are non-specific and are of limited value in practical analysis unless the samples examined are of known pesticide history or have been selectively extracted and cleaned-up. In this laboratory, where samples of unknown history are frequently encountered, we have been studying spray reagents with the object of improving specificity. Our approach to the problem has been to carry out hydrolysis reactions on the thin-layer plate and to use specific or semi-specific colour reactions of the breakdown products produced as an aid in the characterisation of the parent pesticides. COHA[6] has recently applied a similar technique to the detection of the pesticide dimefox. This paper describes techniques capable of detecting and partially characterising carbamate, thiocarbamate, substituted ureas and some nitrogen containing organophosphorus pesticides at μg levels after on-plate hydrolysis to amines.

Experimental

Choice of hydrolyzing procedure. Mixtures of the various compounds, each at about 10 μg, were separated on silica gel thin-layer plates using suitable solvent systems. The plates were then sprayed with a variety of acid and alkaline reagents capable of effecting hydrolysis. The degree of hydrolysis attained was assessed by subsequent spraying with a ninhydrin solution.

Using the screening procedure described, the most effective spray was found to be a hydriodic acid reagent previously recommended by BARNEY[7] for the hydrolysis of organophosphorus compounds. The most complete hydrolysis was achieved when a second glass plate was clipped over the sprayed plate, followed by heating in an oven, at 180°, for about 30 min; a technique we have previously found useful in the hydrolysis of organophosphorus pesticides[8].

Method

Reagents. Spray 1: 25 ml of hydriodic acid, s.g. 1.7, 25 ml of glacial acetic acid and 50 ml of water. The solution is stable for several weeks.

Spray 2: 5 g of sodium nitrite dissolved in 100 ml of 0.2 *N* hydrochloric acid. Prepare the solution just before use.

Spray 3: 5 g of α-naphthol dissolved in 100 ml of methanol. The solution is stable for several days if refrigerated.

Spray 4: 3 g of ninhydrin dissolved in 95 ml absolute ethanol and 5 ml of glacial acetic acid. The solution is stable for several days.

Procedure

The mixture to be separated is divided and the separate portions spotted on to a 20 × 20 cm thin-layer plate coated with a 250 μ layer of Silica Gel G (Merck). The plate is developed in a suitable solvent system, removed from the tank and the solvent allowed to evaporate. The plate is sprayed with spray 1, a clean glass plate clipped over the sprayed surface and the whole heated in an oven at 180° for 30 min. (The plate should be allowed to stand in a vertical plane to assist heat flow over its surface). The plate is removed from the oven and the cover plate unclipped avoiding the iodine fumes evolved. When cool, one portion of the plate is sprayed with spray 2 and gently warmed (*e.g.* on the surface of an oven) until dry. A pink spot on spraying with spray 3 indicates the presence of an aromatic amine. The second portion of the plate is sprayed with spray 4 and heated in an oven at 120° for 20 min. The formation of mauve or reddish brown spots indicates the presence of aromatic or aliphatic amines.

TABLE I

R_F VALUES, COLOURS AND DETECTION LIMITS OF CARBAMATES, UREAS AND ORGANOPHOSPHORUS PESTICIDES AFTER HYDROLYSIS WITH HYDRIODIC ACID

Solvent 1 : hexane–acetone 5 : 1 ; solvent 2 : chloroform–acetone 9 : 1.

*Pesticide**	R_F *solvent 1*	R_F *solvent 2*	*Colour with ninhydrin spray*	*Detection limit* μg	*Colour*** *with diazotising spray*	*Detection limit* μg
Arprocarb	0.24	0.83	brownish red	0.5	—	
Barban	0.33	0.96	pink	5.0	orange-pink	1.0
Butacarb	0.50	0.92	brownish red	0.5	—	
Carbaryl	0.18	0.80	brownish red	0.5	—	
Chlorbufam	0.49	0.92	mauve	2.0	orange-pink	0.5
Chlorpropham	0.65	0.97	mauve	2.0	orange-pink	0.5
Di-allate	0.92	0.95	orange	1.0	—	
EPTC	0.92	0.97	mauve	1.0	—	
Propham	0.53	0.92	mauve	1.0	pink-red	0.5
Tri-allate	0.91	0.95	orange	1.0	—	
Chloroxuron	0.08	0.63	brownish red	0.5	red-mauve	0.5
Cycluron	0.10	0.63	pink	1.0	—	
Diuron	0.12	0.63	brownish red	0.5	orange-pink	0.5
Fenuron	0.11	0.53	brownish red	0.5	pink-red	0.5
Linuron	0.30	0.89	mauve	0.5	orange-pink	0.5
Monuron	0.09	0.48	pink	0.5	pink	0.5
Crufomate	0.06	0.43	brown-red	0.5	—	
Dimefox	0.08	0.44	brown-red	0.2	—	
Dimethoate	0.05	0.37	brown-red	1.0	—	
Ethoate methyl	0.07	0.61	pink	1.0	—	
Fenitrothion	0.49	0.91	mauve	1.0	pink-mauve	1.0
Formothion	0.15	0.75	brownish red	2.0	—	
Mecarbam	0.42	0.95	pink	5.0	—	
Menazon	0.0	0.02	pink	1.0	—	
Morphothion	0.06	0.49	mauve	0.5	—	
Parathion	0.57	0.91	mauve	1.0	pink-mauve	2.0
Phosphamidon	0.04	0.34	brownish red	5.0	—	
Schradan	0.0	0.02	brownish red	0.1	—	
Thionazin	0.45	0.92	brownish red	1.0	—	
Vamidothion	0.01	0.16	brownish red	1.0	—	

* B.S.I. approved names used where applicable.
** —: Indicates no reaction.

Results

Table I shows the R_F values in two solvent systems, detection limits and characteristic colours obtained for a variety of carbamates, substituted ureas and organophosphorus pesticides after hydrolysis with hydriodic acid. The oxygen analogues of dimethoate, parathion, and thionazin gave analogous colour reactions to the parent compounds. The following nitrogenous compounds failed to give colour reactions under the described conditions.

Triazines: ametryne, atrazine, desmetryne, GS 14260, methoprotryne, prometryne and simazine.

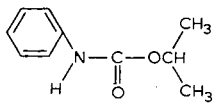
Uracils: bromacil, isocil and lenacil.

Organophosphorus compounds: azinphos ethyl, azinphos methyl, diazinon, phosalone and pyrimithate.

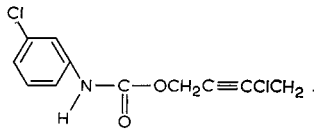
Discussion

The results in Table I, together with our previous work[8] in which hydriodic acid was found to hydrolyze most organophosphorus pesticides to orthophosphate on silica gel layers, indicates the utility of this reagent. In practice we have found the reagent to have the following disadvantages.

(a) The breakdown with hydriodic acid is apparently a stepwise process and some of the detectable breakdown products are not necessarily the ultimate products of the reaction. Thus aromatic amines derived, for example, from carbamates such as propham or barban, and detectable with the chromogenic reagents described, will

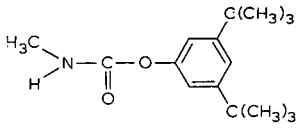


Propham

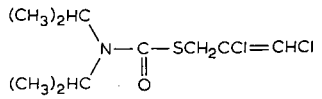


Barban

further decompose to yield products which are not detectable. An optimum heating time at 180° of about 20–30 min was found for such compounds with additional heating leading to a reduction in sensitivity. Carbamates such as butacarb or di-allate



Butacarb



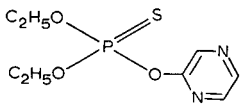
Di-allate

which decompose to aliphatic amines were not subject to such critical sensitivity–time relationships and heating times > 30 min led to enhanced sensitivity.

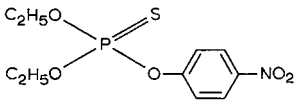
(b) During the heating process iodine fumes are evolved which can react with co-extractives present on the thin-layer plate to give dark areas obscuring any subsequent colour reaction. Where such interference is encountered a suitable clean-up procedure is essential. In our experience river water extracts rarely require clean-up.

Subject to the limitations described we have found the technique useful and reproducible for the thin-layer detection of small quantities of the compounds listed. The nature of the breakdown products produced *i.e.* whether aliphatic or aromatic

amine, the ninhydrin colour (which is frequently characteristic), together with the R_F data all assist in the characterisation of the parent compound. It is possible to distinguish organophosphorus pesticides from carbamates and ureas by use of the phosphate—specific spray described elsewhere[8]. In the interpretation of the thin-layer data caution is necessary as amine formation may not be due merely to simple hydrolysis but may involve ring cleavage or the reduction of nitro groups as is the case with thionazin and parathion respectively.



Thionazin



Parathion

A particularly useful feature of the spray described is the high sensitivity to the compounds dimefox and schradan, organophosphorus pesticides which are frequently difficult to detect by other procedures.

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THEORY OF TRANSPORT IN LINEAR PARTITION SYSTEMS OCCURRING IN CHROMATOGRAPHIC AND ELECTROPHORETIC MODELS

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SUMMARY

General formulae for the transport of matter, under the influence of electric or dynamic forces and which distributes linearly between two states, are derived without any restriction about the nature of the partition process. The moments of the concentration profile are calculated from the formulae. In those moments each term is physically clear. From the results the already known formulae for specialized cases of the partition process can easily be derived.

INTRODUCTION

The following expression governs the transport of matter under influence of flow and diffusion in the unidimensional case

$$\left(\frac{\partial c}{\partial t}\right)_x = -v\cdot\left(\frac{\partial c}{\partial x}\right)_t + D\cdot\left(\frac{\partial^2 c}{\partial x^2}\right)_t \qquad (1)$$

or in short notation

$$\partial_t c = -v\cdot\partial_x c + D\cdot\partial_{xx} c$$

where v is the velocity of the substance. The movement of the substance may be caused by displacement of the fluid in which the substance is dissolved as is the case in chromatography, or by electric forces as in electrophoresis.

The other symbols are as follows:
c = concentration of the substance = $c(x,t)$,
D = diffusion constant; D is assumed to be a constant.

In chromatography and electrophoresis the moving substance is distributed between a "free" and "bound" state. Distribution may result from the presence of a stabilizing medium to which the substance is sorbed; complex formation with a second solute in excess is another possibility. In all these cases the partition process is assumed to be quasi-linear, which means that (in the absence of transport and

diffusion) the concentrations f and b in the free and bound state respectively, are governed by

$$\partial_t f = -l_1 f + l_2 b \tag{2}$$

$$\partial_t b = + l_1 f - l_2 b = -\partial_t f \tag{3}$$

where l_1 and l_2 are the mass transfer coefficients.

With

$$\tau = (l_1 + l_2)^{-1} \tag{4}$$

and the initial condition $f = f_0$, $b = b_0$ we have

$$f = \left(\frac{1}{1+\gamma}\right)(f_0 + b_0) + \tau(l_1 f_0 - l_2 b_0)\cdot e^{-\frac{t}{\tau}} \tag{5}$$

$$b = \left(\frac{\gamma}{1+\gamma}\right)(f_0 + b_0) - \tau(l_1 f_0 - l_2 b_0)\cdot e^{-\frac{t}{\tau}} \tag{6}$$

where the partition coefficient

$$\gamma = \frac{l_1}{l_2} \tag{7}$$

defines the equilibrium state, which is approached exponentially with a time constant τ, while $f + b$ always equals $f_0 + b_0$.

On adding this type of partition term to equation (1), the following equations for the combination of streaming, diffusion and partition are obtained:

$$\partial_t f = -v_f\cdot\partial_x f - (l_1 f - l_2 b) + D_f\cdot\partial_{xx} f \tag{8}$$

$$\partial_t b = -v_b\cdot\partial_x b + (l_1 f - l_2 b) + D_b\cdot\partial_{xx} b \tag{9}$$

When there is only one state f and no diffusion or partition, it can easily be seen that the concentration peak will move with a velocity v_f, because $f(x - v_f t)$ is a solution of the differential equation

$$\partial_t f = -v_f\cdot\partial_x f\,.$$

From physical considerations it is clear, that after some initial effects the mass centre of $(f+b)$ will move uniformly with a velocity $\bar{v}$.

We will now transform to a new coordinate system moving with this velocity, by putting $x' = x - \bar{v}\cdot t$. This makes the mass centre stay at rest with respect to this new system. (A proper choice of the origin of the coordinate system is of course this mass centre.) So

$$\partial_x f = \partial_{x'} f' \tag{10}$$

$$\partial_t f' = \partial_t f + \bar{v}\cdot\partial_x f \tag{11}$$

with similar equations for b' and b.

Substitution in eqns. (8) and (9) gives, on omitting primes:

$$\partial_t f = (\bar{v} - v_f)\cdot\partial_x f - (l_1 f - l_2 b) + D_f\cdot\partial_{xx} f \tag{12}$$

$$\partial_t b = (\bar{v} - v_b)\cdot\partial_x b + (l_1 f - l_2 b) + D_b\cdot\partial_{xx} b \tag{13}$$

The assumption of linearity is quite correct even in the presence of a stabilizing medium if partition takes place in the homogeneous phase inside the stabilizing medium. The paper electrophoretic separation of metal ions in a solution of citric acid in excess is a example of such a system.

In chromatographic columns the stabilizing medium itself participates in the partition process. In these heterogeneous systems the assumption of linearity is only approximately fulfilled. This can only lead to results more or less correct depending on the geometry of the system. Furthermore the equations do not include the statistical variations of the transport velocities caused by the grain or fibre structure of the stabilizing medium.

Temperature fluctuations arising from electric resistance differences or from sorption reactions are also neglected, just like micro-scale gravitational turbulences, etc.

Thus, exact solutions to the equations (12) and (13) are of limited value as the equations themselves are open to criticism. Moreover, we get more information than required for practice. We are interested in quantity, displacement, width and skewness of the mass distribution $(f+b)$ and the intention is to deduce these variables from the measured signals. These signals are obtained either as a function of time (elution chromatography, gas chromatography) or as a function of place (electrophoresis, thin layer chromatography). There is a relation between the above mentioned variables and the moments of the signals which are defined by the partial integral:

$M_i = \int \xi^i \cdot S(\xi) \cdot \mathrm{d}\xi$, where ξ is either the time or a coordinate depending on the experimental conditions. These moments are a—preferably linear—function of the respective identically defined moments of the concentration distribution; this function is determined by the instrumental conditions. The moments of the concentration distribution are linear with respect to the above mentioned variables.

Several authors have shown that it is possible to obtain the moments of the concentration distribution directly from equations (12) and (13), and depending on their inclination they calculate either the time moments[1–3] or the place moments[4–8].

The time and place moments are of course strongly related to each other, as the separation process itself is independent of the method of definition and measurement. The mutual relationship will be discussed later in this paper. Although there is no fundamental difference between the two types of moments, we chose to calculate the place moments as during the calculating procedure the physical meaning is better. The place moments are yielded directly by integration of eqns. (12) and (13). It will be shown that specification of the partition process is not necessary. Only LJUNGGREN[4] has attempted to calculate the place moments in a similar way but he either neglected the thermal diffusion or the electric field. It will be shown that during the calculations the essence of the separation process can be followed step by step and the physical meaning and origin of each term can be easily understood.

DEFINITIONS AND MEANING OF MOMENTS

The separating system consists of a column of infinite length with a concentration peak of arbitrary form localized in a small region.

The ith moments of the concentration distribution are defined by:

$$F_i = \int x^i \cdot f \cdot \mathrm{d}x \text{ and } B_i = \int x^i \cdot b \cdot \mathrm{d}x \qquad (14)$$

We then have

F_0 is the total amount of substance in the free state; F_0 is a function of time only;
B_0 is the total amount in the bound state;
F_1 defines the centre of mass in the usual way.
In addition, we define the coordinate x_f of this mass centre by:

$$F_1 = x_f \cdot F_0 \tag{15}$$

Similar relations hold for B_1.

It is also well known that the variance μ is indicative for the width of a distribution and that this quantity is defined by

$$\mu_f = \frac{\int (x - x_f)^2 \cdot f \cdot \mathrm{d}x}{\int f \cdot \mathrm{d}x} \tag{16}$$

which makes

$$\mu_f = \frac{F_2}{F_0} - \left(\frac{F_1}{F_0}\right)^2 = \frac{F_2}{F_0} - x_f^2 \tag{17}$$

Analogous relations apply to μ_b and B_2.

The third moments F_3 and B_3 are related to the skewness of the concentration peaks. The discussion of these quantities is given in the corresponding section of this paper.

We shall now calculate the ith moments. To do so we need the following equations derived from the definition (14):

$$\int x^i \cdot \partial_t f \cdot \mathrm{d}x = \mathrm{d}_t F_i \tag{18}$$

$$\int x^i \cdot \partial_x f \cdot \mathrm{d}x = \begin{cases} -iF_{i-1} & (i = 1, 2 \ldots) \\ 0 & (i = 0) \end{cases} \tag{19}$$

$$\int x^i \cdot \partial_{xx} f \cdot \mathrm{d}x = \begin{cases} i(i-1)F_{i-2} & (i = 2, 3 \ldots) \\ 0 & (i = 0, 1) \end{cases} \tag{20}$$

Similar relations hold for the bound state b.

CALCULATION OF THE MOMENTS

The zeroth moment

Integrating (12) and (13) along the x-axis using eqns. (18), (19) and (20) we obtain

$$\mathrm{d}_t F_0 = -(l_1 F_0 - l_2 B_0) \tag{21}$$

$$\mathrm{d}_t B_0 = +(l_1 F_0 - l_2 B_0) \tag{22}$$

because only the partition terms contribute. This would be expected, as the chemical reaction is the only way of changing the amount of substance in each state.

As (21) and (22) are identical with (2) and (3), with initial conditions $F_0 = F_{00}$, $B_0 = B_{00}$ and inserting $c = l_1 F_{00} - l_2 B_{00}$ and

$$F_{00} + B_{00} = M_0 \tag{23}$$

we have

$$F_0 = \left(\frac{1}{1+\gamma}\right) M_0 - c\tau \cdot e^{-\frac{t}{\tau}} \tag{24}$$

$$B_0 = \left(\frac{\gamma}{1+\gamma}\right) M_0 + c\tau \cdot e^{-\frac{t}{\tau}} \tag{25}$$

In fact in the partition-only case of (2) and (3)

$$f_0 \mathrel{\hat{=}} F_{00} \text{ and } b_0 \mathrel{\hat{=}} B_{00}$$

From these equations we conclude that transport and diffusion have no influence on partition. The steady state is exponentially approached with a time constant τ and in practice can be said to be reached after 4τ.

Note that in electrophoresis the electric field is only established after injection of the sample; so the constant c can be made zero and the steady state is immediately reached.

The first moment

On multiplying (12) and (13) by x and integrating along the x-axis we find that as a result of (20) the diffusion term does not contribute. We then have

$$d_t F_1 = (v_f - \bar{v}) F_0 - (l_1 F_1 - l_2 B_1) \tag{26}$$

$$d_t B_1 = (v_b - \bar{v}) B_0 + (l_1 F_1 - l_2 B_1) \tag{27}$$

With

$$M_1 = F_1 + B_1 = x_{fb} \cdot M_0 \tag{28}$$

we obtain from these equations for the velocity v_{fb} of the mass centre of the total amount of substance

$$v_{fb} = d_t x_{fb} = \left(\frac{v_f + \gamma v_b}{1+\gamma}\right) - \bar{v} + (v_b - v_f) \frac{c\tau}{M_0} \cdot e^{-\frac{t}{\tau}} \tag{29}$$

So from the definition of $\bar{v}$ in the coordinate transformation it follows that $\bar{v}$ is equal to the weighted arithmetic mean of v_f and v_b:

$$\bar{v} = \left(\frac{1}{1+\gamma}\right) v_f + \left(\frac{\gamma}{1+\gamma}\right) v_b \tag{30}$$

It follows from (29), remembering that the origin of the moving coordinate system should be at the mass centre, after the disappearance of the initial effects that:

$$x_{fb} = (v_f - v_b) \frac{c\tau^2}{M_0} \cdot e^{-\frac{t}{\tau}} \tag{31}$$

All further derivations will be made in moving coordinates only. To find the values of F_1 and B_1 we multiply (26) by l_1 and (27) by l_2 and subtract the equations. From this we get after integrating*:

$$l_1 F_1 - l_2 B_1 = \left(\frac{\gamma}{1+\gamma}\right) \left(\frac{1}{1+\gamma}\right) (v_f - v_b) M_0 + (d_1 + d_2 \cdot t) \cdot e^{-\frac{t}{\tau}} \tag{32}$$

Combining this equation with (15), (24) and (28) we obtain for the separate mass centres of the free and the bound states:

* d_1, d_3, d_5, f_1 and f_3 are constants determined by the initial conditions, d_2, d_4, d_6, f_2 and f_4 are constants determined by the integration.

$$x_f = \left(\frac{\gamma}{1+\gamma}\right)(v_f - v_b)\tau + (d_3 + d_4 t)\,e^{-\frac{t}{\tau}} \tag{33}$$

$$x_b = -\left(\frac{1}{1+\gamma}\right)(v_f - v_b)\tau + (d_5 + d_6 t)\,e^{-\frac{t}{\tau}} \tag{34}$$

After disappearance of the exponential terms, which quickly occurs, we have:

$$\Delta x = x_f - x_b = (v_f - v_b)\tau \tag{35}$$

$$x_f = (v_f - \bar{v})\tau \tag{36}$$

$$x_b = (v_b - \bar{v})\tau \tag{37}$$

and not withstanding:

$$x_{fb} = 0\,.$$

The shift of the mass centres with respect to each other, acquired during the lifetime of the exponentials and the shift towards the origin are noteworthy. The shifts arise from the fact that when a molecule moves in state "f" its velocity in moving coordinates equals $(v_f - \bar{v})$. The mean time for a molecule to be either in state "f" or in state "b" is equal to τ. So we can understand both x_f and x_b as the mean displacement of one molecule. These are equal to the displacements of the mass centres of the ensembles of molecules.

The second moment

Following the same procedure as for F_1 and B_1 we get for the second moment:

$$d_t F_2 = 2(v_f - \bar{v})F_1 - (l_1 F_2 - l_2 B_2) + 2D_f F_0 \tag{38}$$

$$d_t B_2 = 2(v_b - \bar{v})B_1 + (l_1 F_2 - l_2 B_2) + 2D_b B_0 \tag{39}$$

With $M_2 = F_2 + B_2$ we obtain after adding the equations:

$$\begin{aligned} d_t M_2 = {} & 2\left\{\left(\frac{1}{1+\gamma}\right)D_f + \left(\frac{\gamma}{1+\gamma}\right)D_b\right\} M_0 + \\ & + \frac{2}{\tau}\left\{\left(\frac{1}{1+\gamma}\right)(v_f - \bar{v})^2\tau^2 + \left(\frac{\gamma}{1+\gamma}\right)(v_b - \bar{v})^2\tau^2\right\} M_0 + \\ & + (f_1 + f_2 t)\,e^{-\frac{t}{\tau}} + (f_3 + f_4 t)\,e^{-\frac{2t}{\tau}} \end{aligned} \tag{40}$$

Applying (17) we obtain for the variance of the total amount of substance:

$$\mu = \frac{M_2}{M_0} - x_{fb}^2 \tag{41}$$

On integrating (40) and cancelling of the exponentials

$$\mu = \mu_0 + 2\left\{\left(\frac{1}{1+\gamma}\right)D_f + \left(\frac{\gamma}{1+\gamma}\right)D_b\right\}t + 2\left\{\left(\frac{1}{1+\gamma}\right)x_f^2 + \left(\frac{\gamma}{1+\gamma}\right)x_b^2\right\}\frac{t}{\tau}$$

$$= \mu_0 + 2\left[\bar{D} + \frac{1}{\tau}\left\{-x_f \cdot x_b\right\}\right]t$$

$$= \mu_0 + 2\left[\bar{D} + \left(\frac{1}{1+\gamma}\right)\left(\frac{\gamma}{1+\gamma}\right)(v_f - v_b)^2\tau\right]t$$

$$= \mu_0 + 2D' \cdot t \tag{42}$$

In this case μ_0 is the initial variance determined by the width of the concentration peak at $t=0$. Note that $\mu \neq \mu_0$ at $t=0$ according to the exponentials; their contribution arises from a combination of the mass centre shift away from the origin and a mass partition unequal to γ.

From (42) it is clear that the peak is broadened by diffusion with a coefficient $\bar{D}$ equal to the weighted mean of D_f and D_b. In addition to diffusion there is another broadening effect proportional to the reaction time constant τ. This contribution arises from the kinetics of the separation process and has its maximum when $\gamma = 1$ and is zero for $\gamma = 0$ and infinity. The contribution can be understood from a random walk model[9]: If y is the mean length of a step forward and z is the mean length of a step backwards and m and n are the mean number of steps then the displacement Δ is given by:

$$\overline{\Delta^2} = m \cdot y^2 + n \cdot z^2 \tag{43}$$

(Assuming $my + nz = 0$). In our case the total number of steps will be t/τ as τ is the mean time to be in each state. Correspondingly the number of steps of one molecule in state f is $m = (1/1+\gamma)\,(t/\tau)$ and so $n = (\gamma/1+\gamma)\,(t/\tau)$. Substitution leads to the contributing term.

The third moment

The third moment M_3 is related to the skewness. From mathematical statistics[11] we know that when a distribution approximates a normal distribution the following equation holds relating the Pearson measure of skewness[12] $(x_{fb} - x_{max})/\sigma$ to $S = M_3/M_0 = (F_3 + B_3)M_0$ (x_{max} = the coordinate for which the distribution has its maximum, $\sigma = \sqrt{\mu}$ = standard deviation):

$$\frac{(x_{fb} - x_{max})}{\sigma} = \frac{S}{2\sigma^3} \tag{44}$$

So:

$$(x_{fb} - x_{max}) = \frac{S}{2\mu} \tag{45}$$

To determine the third moment we derive:

$$d_t F_3 = 3(v_f - \bar{v})F_2 - (l_1 F_3 - l_2 B_3) + 6D_f F_1 \tag{46}$$

$$d_t B_3 = 3(v_b - \bar{v})B_2 + (l_1 F_3 - l_2 B_3) + 6D_b B_1 \tag{47}$$

and so by addition:

$$d_t M_3 = 3\{(v_f - \bar{v})F_2 + (v_b - \bar{v})B_2\} + 6(D_f F_1 + D_b B_1)$$

$$= 3(v_f - v_b)\tau\{l_1 F_2 - l_2 B_2\} + 6(D_f F_1 + D_b B_1) \quad (48)$$

On substituting known F's and B's:

$$d_t M_3 = 6\left(\frac{1}{1+\gamma}\right)\left(\frac{\gamma}{1+\gamma}\right)(v_f - v_b)\tau M_0\left\{\frac{x_f^2 - x_b^2}{\tau} + 2(D_f - D_b)\right\} + \text{exponentials} \quad (49)$$

Integrating and cancelling of the exponentials gives

$$S = S_0 + \left\{12(v_f - v_b)\tau\left(\frac{1}{1+\gamma}\right)\left(\frac{\gamma}{1+\gamma}\right)(D_f - D_b) + \right.$$

$$\left. + 6(v_f - v_b)^3\tau^2\left(\frac{1}{1+\gamma}\right)\left(\frac{\gamma}{1+\gamma}\right)\left(\frac{\gamma-1}{\gamma+1}\right)\right\}\cdot t \quad (50)$$

S is changing linearly with time. The first time-dependant term is a transport-diffusion mixture term, which has its maximum for $\gamma = 1$. The second one is a transport–partition term with a maximum for $\gamma = 3.7$ and $1/3.7$ and a zero value for $\gamma = 1$. We can substitute (50) in (45) and get something like:

$$x_{fb} - x_{\max} = \frac{(S_0 + \beta \cdot t)}{2(\mu_0 + 2D't)} \quad (51)$$

In most practical cases $S_0 \approx 0$ and so $(x_{fb} - x_{\max})$ rises from zero to an asymptotic time-independent value $(\beta/4D')$, assuming that the concentration distribution is approximately Gaussian. The magnitude of this asymptotic value will be investigated in two extreme cases:

(a) The diffusion determines the width of the concentration profile. Then $(v_f - v_b)^2\tau \ll \bar{D}$ and

$$\frac{\beta}{4D'} = \frac{3\left(\frac{1}{1+\gamma}\right)\left(\frac{\gamma}{1+\gamma}\right)(D_f - D_b)(v_f - v_b)\tau}{\bar{D}} \quad (52)$$

The latter function for $D_b = 0$ and $\gamma \gg 1$ reaches the extreme

$$\frac{\beta}{4D'} = 3(v_f - v_b)\tau = 3(x_f - x_b) \quad (53)$$

(b) When $(v_f - v_b)^2\tau \gg \bar{D}$ the asymptotic value transforms in

$$\frac{\beta}{4D'} = \frac{3}{2}\left(\frac{\gamma-1}{\gamma+1}\right)(v_f - v_b)\tau \quad (54)$$

with an extreme value of

$$\frac{\beta}{4D'} = \frac{3}{2}(v_f - v_b)\tau = \frac{3}{2}(x_f - x_b) \quad (55)$$

The importance of these derivations is that the maximum distance between maximum and mean of the concentration profile is comparable with the shift of the mass centres f and b during the separation process. The skewness $(x_{fb} - x_{\max})/\sigma$ diminishes with respect to the root of the time, as it should, which is in agreement with the computer results of VINK[6] for his partition model.

DISCUSSION

We can now compare the results with formulae published in other papers.

LJUNGGREN[4] made a theoretical study of electrodiffusion by means of moment analysis. He solved the equations after introduction of the moments by Fourier and Laplace transformations but he either neglected the ordinary diffusion or the electric field. He, nevertheless, derived a partition–transport contribution to the diffusion term which agrees with the third term in eqn. (42).

In chromatography there is the restriction $v_b = 0$ and in addition, our partition coefficient is related to R by $\gamma = R/1-R$. Substitution transform the partition–transport term in the well known result[10]

$$\Delta\mu = 2R(1-R)\cdot v\cdot t_a\cdot L \tag{56}$$

VINK[5] made a chromatographic model in which the partition occurs between two rows of unit cells of unequal volume per unit of contact area and separated by a membrane. In this model the partition is controlled by the diffusion in the bound state b. This makes the kinetic constants equal to

$$l_1 = \frac{2D_2}{V_2\cdot V_1}\cdot\gamma \text{ and } l_2 = \left(\frac{2D_2}{V_1\cdot V_2}\right)\cdot\left(\frac{V_1}{V_2}\right)$$

Substitution shows agreement between his formulae[7] and equation (42). The first and third term arise from the weighted mean of the diffusion coefficients; the second term in his formulae (34) originates from the exchange between partition and transport.

In KUČERA's paper[1], the model is assumed to consist of a column filled with small porous grains. In this model the partition occurs in two steps, *viz.* diffusion through the pores followed by sorption on the internal surfaces. First of all confining our attention to the calculation of the time-moments in this paper, and neglecting for a while the extension to a third "phase", we should expect the relation between the ith time and ith place moment to be simply

$${}_i\mu_x = (\bar{v})^i\cdot{}_i\mu_t$$

In this case, however, the broadening of the distribution by diffusion and other effects is neglected during the time the distribution passes the fixed place $x = L$. An impression of the correction for this is obtained by dividing a normal distribution into two parts and assuming that the mass points of each part of the distribution lie at distances $+\sigma$ and $-\sigma$ from the intersection. Now the elution time for the peak will be $\Delta t = 2\sigma/\bar{v}$. During this time the distance 2σ grows according to $\sigma^2 = 2D'\cdot t$ with

$$2\Delta\sigma = \frac{2D'}{\sigma}\cdot\Delta t = \frac{4D'}{\bar{v}}$$

Half the mass shifts this distance making the shift of the mass point of the whole distribution equal to $2D'/\bar{v}$ or in time coordinates $\Delta t' = 2D'/\bar{v}^2$. As the curve is lagging behind the time discrepancy has to be added, giving:

$${}_1\mu_t = \frac{L}{\bar{v}} + \frac{2D'}{\bar{v}^2} \tag{57}$$

The additional term for the second moment originates from the same effect. The shift in distance relative to the central mass point is $2D'/\bar{v}$ for each part of the curve. So the contribution to ${}_2\mu_x$ will be $(2D'/\bar{v})^2$. Substitution gives:

$$ {}_2\mu_t = {}_2\mu_x/\bar{v}^2 + (2D'/\bar{v})^2/\bar{v}^2 = \frac{2D'}{\bar{v}^2}\left(\frac{L}{\bar{v}} + \frac{2D'}{\bar{v}^2}\right) + \frac{4D'^2}{\bar{v}^4} = \frac{2D'L}{\bar{v}^3} + \frac{8D'^2}{\bar{v}^4} \qquad (58) $$

Both equations agree with the results of KUČERA[1]. The calculations here are of course not exact but they give an idea of the origin and the physical meaning of the several terms in the time moments.

KUBIN[2] obtained some different results on calculating the same time moments. The discrepancy arises from his initial conditions. He injects a block function with a time width t_0, which gives a contribution to the first moment of $\frac{1}{2}t_0$ as a consequence of the mass point shift relative to the origin of the coordinate system. For the same reason the second moment is extended by $\frac{1}{12}t_0^2$ (the inertia moment of a rectangular box is $\frac{1}{12}ml^2$). The distortion contributions of KUČERA are lost by an approximation in the calculation.

We can now look at what happens when the number of states is extended. For three states we have:

$$ \delta_t f = (\bar{v} - v_f)\cdot\delta_x f + D_f\cdot\delta_{xx} f - (l_{12}f - l_{21}b) - (l_{13}f - l_{31}d) \qquad (59) $$

$$ \delta_t b = (\bar{v} - v_b)\cdot\delta_x b + D_b\cdot\delta_{xx} b - (l_{21}b - l_{12}f) - (l_{23}b - l_{32}d) \qquad (60) $$

$$ \delta_t d = (\bar{v} - v_d)\cdot\delta_x d + D_d\cdot\delta_{xx} d - (l_{31}d - l_{13}f) - (l_{32}d - l_{23}b) \qquad (61) $$

This system extends by one equation, and simultaneously each equation by one exchange term, for each additional state. Similarly to the case with two states, both the time constant τ and the factors α_i determining the partition of total mass M_0 over the three states are only a function of the kinetic constants l. In general, it is a complicated function when both side and successive reactions exist. An important conclusion is that there is only one time constant τ for the whole system.

A characteristic fact from the equations (59)–(61) is that the sum of all kinetic terms is zero. So they always vanish in the calculation of M_i. Consequently from equations (26) and (27) it follows

$$ \mathrm{d}_t M_1 = (v_f - \bar{v})F_0 + (v_b - \bar{v})B_0 + (v_d - \bar{v})D_0 \qquad (62) $$

This equation is familiar to us from mechanics as it follows immediately from the definition of mass point.

Equation (30) changes in the general case into

$$ \bar{v} = \alpha_f\cdot v_f + \alpha_b\cdot v_b + \alpha_d\cdot v_d + \ldots \qquad (63) $$

In chromatography all velocities are zero except v_f making the reduced velocity simply equal to the fraction of solute in the mobile phase. This can be stated independently of the column model and is a fundamental law of chromatography[8].

After introduction of the moving coordinate system we get for the steady state similar to before: $d_t M_1 = 0$, $x_{fb} = 0$, F_0, B_0 etc. = constant. This implies that apart from an exponential term the terms $(l_{ij}B_1 - l_{ji}D_1)$, etc. occurring in the extended form of equation (26) will not contain the terms of any order in t; this agrees with equation (32). So $d_t F_1 = d_t B_1 = \ldots = 0$. By comparing the procedure for solution of F_0 and F_1 combined with dimensional analysis it becomes clear how the time constant τ depends on the l-values and how τ enters the equations (33) and (34).

It can be verified that equations analogous to (33) and (34) can be derived and from these it can be deduced that the shift of the mass centre of each state is the same as was found before (equations (36) and (37)). So the equation

$$x_i = (v_i - \bar{v})\tau \tag{64}$$

may be stated as a general equation.

In the same way as before we can derive the time derivative of the second moment M_2. The equation is nearly the same as equation (40). On integrating and cancelling of the exponentials we get an equation like (42). So in general the width of the distribution will be equal to:

$$\mu = u_0 + 2D' \cdot t \tag{65}$$

in which

$$D' = \sum_i a_i D_i + \frac{1}{\tau} \sum_i a_i x_i^2 \tag{66}$$

the summation takes place over all states. A general conclusion now is that:

The laws governing the behaviour of the zeroth, first and second moments can be stated independently of the model and are to be considered as fundamental laws.

With the derived formulae it was possible to verify the general results of KUČERA[1], for the three state model.

VINK[8] defined a model which was extended to n states by induction. His formulae for the displacement and width can be derived from the general formulae. The formulae are also applicable to frontal analysis as was shown by VINK[8] by taking the place derivatives.

So far transportation space has been treated as if it were devoid of structure. Such an assumption is certainly wrong in paper electrophoresis and chromatographic columns. In this case homogeneity is disturbed and the velocities become functions of x, y, z. The nature of the stabilizing medium does not permit a definite statement of these functions. The problem is approximated in a statistical way. This means that:

(a) v_f and v_b are fluctuating statistically;

(b) the mean values for v_f and v_b are lowered differently by obstructivity and tortuosity of the substrate.

The influence on the previously derived results of this is:

(a) one more term contributing to the formula for the width of the concentration peak;

(b) slowing down of the mean transport.

A contribution to the skewness is difficult to assess. If there is any contribution at all, it is probably small.

There is still one remarkable thing. It may occur, that a solute divides between

two states in which the velocities are opposite. In that case $\bar{v}$ will be small, but the solute particles are travelling back and forth. x_i from equation (64) is the mean travelling distance in state i. When the time constant τ is large, x_i may become larger than the dimensions of the grains and fibres of the stabilizing medium. In this case the spreading effects may be excessively large, whereas in the opposite case there is no influence at all. This problem may occur in electrophoresis. In chromatography the problem does not exist as all velocities have the same sign.

CONCLUSION

It is shown that it is possible to solve the general formulae describing the transport of matter under the influence of electric or dynamic forces and distributing itself linearly between several states. Each term in the formulae for the calculated moments has a distinct physical meaning. The results for the first and the second moment are simple and it is easy to extend the formulae to systems in which one component is distributed between more than two states. For the calculation of the mean velocity and the width we need to know the fraction α of the solute in each state and the time constant τ of the system. If the separate reaction velocity constants l_{ij} are not known, as is common, the α_i's may be determined by chemical analysis or in a more sophisticated way together with τ from N.M.R. or E.S.R. experiments. It is in general not necessary to evaluate them from chromatographic or electrophoretic experiments. There is one thing still to note, being the effect on the separation process of taking away the driving forces at the end of a thin-layer chromatographic or an electrophoretic separation. It is easy to understand that the system proceeds in an exponential way to the state in which the mass centres of the free and the bound state(s) coincide, while the distribution as a whole stays at rest. From then on, broadening of the concentration distribution only arises from diffusion.

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IDENTIFIZIERUNG GAS-CHROMATOGRAPHISCH GETRENNTER ALKOHOLE MITTELS DEHYDRIERUNG ZU CARBONYLVERBINDUNGEN

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SUMMARY

Identification by dehydrogenation to carbonyl compounds of alcohols separated by gas chromatography

The alcohols were separated in argon carrier gas, which contained 5 % hydrogen, and dehydrogenated in a post-column reactor. In this way primary and secondary alcohols split off hydrogen and formed carbonyl compounds. Tertiary alcohols were dehydrated and the olefines formed were saturated. Since the components were completely adsorbed, the detector registered only the hydrogen formed or its absence in the form of negative or positive peaks.

EINLEITUNG

Für die Strukturbestimmung und gruppenmässige Identifizierung komplizierter organischer Stoffe benutzt man oft gas-chromatographische Reaktionsmethoden. Meistens wird die Reaktion von der Analyse durchgeführt, in der Säule werden Reaktionsprodukte getrennt.

Nur wenige Autoren haben die Reaktion erst nach der gaschromatographischen Trennung benutzt, obgleich diese Methode willkommene Kenntnisse zur Identifizierung einzelner Berge darstellt.

Man verfährt so, dass nur flüchtige Reaktionsprodukte detektiert werden, andere werden vollständig adsorbiert. Mit Reaktion auf $NaAlH_4$ ist es möglich festzustellen, welche Komponente aktiven Wasserstoff enthalten[1]. Unsere früheren Arbeiten beschäftigten sich mit katalytischer Hydrogenolyse[2], Bestimmung von Alkoxylgruppen[3] und Sauerstoffverbindungen, die eine gerade Kohlenwasserstoffkette besitzen[4]. In der letzten Arbeit[4] enthielt der Hydrogenolysereaktor, ausser Katalysator, Molekularsiebe. Die entstandenen *n*-Paraffine wurden adsorbiert, den Detektor passierten nur cyclische und verzweigte Reaktionsprodukte.

Neue Möglichkeiten für gruppenmässige Identifizierung getrennter Substanzen bietet Hydrierung und Dehydrierung in einem Zweikomponenten-Trägergas (Wasserstoff + Argon)[5]. Die Bestimmung der Konzentration des Wasserstoffs zeigt, von welchen Komponenten Wasserstoff frei wird und welche ihn verbrauchen. Gleiche

Ergebnisse erhielten LITTLEWOOD UND WISEMAN mit einem electrochemischen Detektor[6].

In diesen Arbeiten[5,6] wurden nur Mischungen von Kohlenwasserstoffen untersucht. Es ist wohlbekannt, dass auch aus einigen Heteroverbindungen in bestimmten Bedingungen Wasserstoff frei wird; es ist möglich Alkohole, Amine und Phenole zu dehydrieren.

In der vorliegenden Untersuchung wurde es festgestellt, in welchem Masse die vorhergenannte Dehydrierungsmethode[5] zur Identifizierung und Strukturbestimmung von Alkoholen brauchbar ist.

THEORETISCHE KALKULATIONEN

Nach der Stellung der Hydroxylgruppe im Molekül unterscheidet man primäre (1), sekundäre (2) und tertiäre (3) Alkohole. Da die primären und sekundären Alkohole das Hydroxyl an einem Kohlenstoffatom enthalten, das ausserdem noch, entsprechend 2 oder 1 Wasserstoffatome zuführt, können sie nach Gleichungen (1) und (2) dehydriert werden.

$$R\text{-}CH_2\text{-}OH \longrightarrow R\text{-}CHO + H_2 \qquad (1)$$

$$\begin{array}{l} R\text{-}CH\text{-}OH \longrightarrow R\text{-}CO + H_2 \\ \quad\;\; | \qquad\qquad\quad\; | \\ \quad\;\; R \qquad\qquad\quad\; R \end{array} \qquad (2)$$

$$\begin{array}{l} \quad\;\; R \qquad\qquad\quad\; R \\ \quad\;\; | \qquad\qquad\quad\; | \\ R\text{-}C\text{-}OH \longrightarrow R = C + H_2O \\ \quad\;\; | \qquad\qquad\quad\; | \\ \quad\;\; R \qquad\qquad\quad\; R \end{array} \qquad (3)$$

Man erhält entsprechende Aldehyde und Ketone. Diese Reaktion in flüssiger Phase benutzt man zur qualitativen Bestimmung der Alkoholen[7], in Dampfphase aber bei 200–300° zur industriellen Synthese von Carbonylverbindungen[8]. In beiden Fällen benutzt man Kupferkatalysatoren. Unter gleichen Bedingungen werden die sekundären Alkohole bedeutend schneller dehydriert als die primären.

Weil die tertiären Alkohole am hydroxyltragenden Kohlenstoffatom kein Wasserstoffatom haben, können sie nicht dehydriert werden. Die Dehydratation nach Gleichung (3) aber geht sehr leicht vor sich; es werden Alkene gebildet. Bei gaschromatographischen Reaktionen werden die tertiären Alkohole immer entwässert[9]. Wenn bei der Katalyse Wasserstoff anwesend ist und ein hydrierender Katalysator angewandt wird, werden die entstandenen Olefine gesättigt. Abspaltung oder Verbrauch von Wasserstoff kann zur Identifikation isomerer Alkohole dienen.

BESCHREIBUNG DER APPARATUR

Der Chromatograph ist sowjetischen Ursprungs UKh, enthält zwei Säulen und ist ein zweckmässiges Apparat für reaktions-gas-chromatographische Arbeiten. Das ursprüngliche Gemisch wurde in einer 6 m langen Säule (Durchmesser 6/4 mm) mit 20 % Polyäthylenglykol 4000 auf Chromosorb W (0.25–0.35 mm) bei 100° getrennt. Der Gasverbrauch war 60 ml pro Min.

Der Hydrierungs–Dehydrierungsreaktor wurde an der Stelle der zweiten Säule eingesetzt. Zu diesem gehörte ein Glasrohr mit Katalysator (1 ml) und, zwecks Erhitzung desselben, ein 20 cm langer Rohrofen. Zwischen dem Reaktor und dem Detektor war eine 25 cm lange, mit Aktivkohle gefüllte Säule eingeschaltet. Von den eingespritzten Stoffen passierten den Detektor nur der bei Dehydrierung erzeugte Wasserstoff oder dessen Vakanzen, verursacht durch Hydrierungsreaktionen, die erstgenannten als negative, die letztgenannten als positive Berge.

Die Eigenart der katalytischen Reaktion ist in bedeutendem Masse von den Eigenschaften des Katalysators abhängig. Die wichtigsten Kennzahlen, die in dieser Arbeit benutzten Katalysatore, sowie ihre Wirkung auf den Ablauf einiger Reaktionen charakterisieren, sind in der Tabelle I angegeben. Platin und Palladium sind wohlbekannte Katalysatoren für Hydrogenation–Dehydrogenation; ihre katalytischen Eigenschaften sind sich ähnlich und hauptsächlich vom Trägermaterial abhängig[10]. Deswegen ist der Katalysator Pd/Kieselguhr bedeutend aktiver als Pt/Chromosorb W. Auf dem ersten werden bei 300° überschreitenden Temperaturen von Sauerstoffverbindungen (Ketone, Alkohole usw.) Heteroatome abgespaltet, auf dem zweiten vollziehen sich hauptsächlich Hydrierungsreaktionen. Kupfer ist ein spezieller Katalysator für Dehydrogenation von Alkoholen.

TABELLE I

EINIGE STRUKTUR- UND WIRKUNGSCHARAKTERISTIKEN[a] VON KATALYSATOREN

Metall	*Träger*	*Konzentration des Metalls*		*Dehydrogenation von Cyclohexan bei 325°*	*Hydrogenation von Olefinen bei 300°*	*Hydrogenolyse von Sauerstoff bei 350°*	*Dehydrogenation von Alkoholen bei 300°*
		%	*mg/cm³*				
Pd	Kieselguhr	5.0	22	+[b]	+	+	+
Pt	Chromosorb W	5.4	13	—	+	—	+
Cu	Chromosorb W	5.0	12	—	—	—	+

[a] Im Trägergas Argon + 5% Wasserstoff.
[b] Positive Reaktion: +; keine Reaktion: —.

Als Trägergas benutzten wir: (1) Wasserstoff, (2) Argon, und (3) 95% Argon + 5% Wasserstoff. Im Wasserstoffstrom wurden Ausgangsgemische analysiert, ebenso die bei der Katalyse entstehenden Kohlenwasserstoffe. Im Argonstrom können nur Dehydrierungsreaktionen ablaufen; anfangs ist der Katalysator sehr aktiv, sogar *n*-Paraffine werden dehydriert[5,11], aber der Katalysator wird schnell vergiftet. Im Gasgemisch Argon + Wasserstoff finden auch Hydrogenationsreaktionen statt. In diesem Gasgemisch werden andere Sauerstoffverbindungen, ausser Alkoholen, nicht dehydriert.

EXPERIMENTE MIT ISOMEREN BUTANOLEN

Die Wirkung der Struktur von Alkoholen auf ihre Dehydrierung ist leicht an den isomeren Butanolen zu beobachten. Es sind: primäres Butanol (*n*-Butanol-1), sekundäres Butanol (*n*-Butanol-2), Isobutanol (2-Methylpropanol-1) und tertiäre Butanol (2-Methylpropanol-2). Dem Butanolgemisch war auch ein Olefin zugefügt

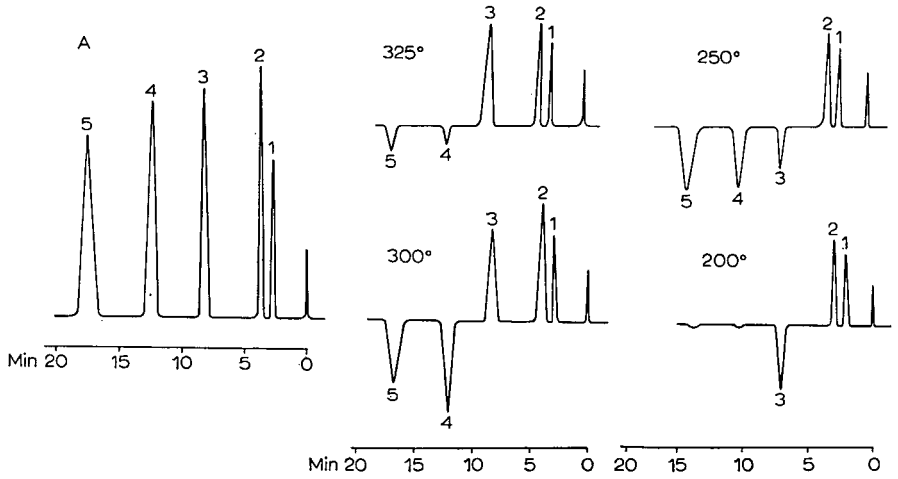


Fig. 1. Untersuchung der Dehydrierungs- und Dehydratationsreaktionen von isomeren Butanolen mit einem der Säule nachgeschalteten Reaktor auf aktivem Katalysator Pd/Kieselguhr. (A) Chromatogramm des ursprünglichen Gemisches, Trägergas H_2. Chromatogramme bei 200° bis 325° stellen die Katalyse bei den entsprechenden Temperaturen dar; Trägergas 95 % Ar, 5 % H_2. Den positiven Bergen entspricht bei der Dehydratation verbrauchter Wasserstoff, den negativen bei der Dehydrierung abgespaltener Wasserstoff. Die Komponenten des Gemisches: (1) *n*-Okten-1 (Vergleichssubstanz); (2) tertiärer Isobutylalkohol; (3) sekundärer *n*-Butylalkohol; (4) primärer Isobutylalkohol; (5) primärer *n*-Butylalkohol.

worden. Wenn Wasserstoff anwesend ist, wird dieser auf hydrierenden Katalysatoren vollständig hydriert und ihr Verbrauch von Wasserstoff kann zum Vergleichsberg dienen. Die Versuche wurden im Temperaturbereich 200–350° durchgeführt und die Menge des von jeden Verbindung abgespaltenen oder gebrauchten Wasserstoffs graphisch dargestellt (Molekül Wasserstoff pro Molekül Verbindung). Auf Fig. 1 sind einige Reaktionschromatogramme dargestellt. Es wurde aktiver Pd-Katalysator benutzt und im H_2 + Ar-Strom chromatographiert. Die quantitative Ergebnisse können wir auf Fig. 2-I sehen.

Bei 280° überschreitenden Temperaturen wird von sekundärem und tertiärem Butanol der Sauerstoff fast vollständig abgespaltet, nur bei niedrigen Temperaturen verläuft die Dehydrierung des sekundären Butanols schneller als seine Dehydratation. Die 1-Butanole reagieren gleich; bei hohen Temperaturen geht ihre Wasserstoffabspaltung und Dehydratation mit gleicher Geschwindigkeit vor sich, deswegen sind die Wasserstoffberge niedrig. Es stellt sich heraus, dass sekundärer Butanol mit grösserer Heftigkeit reagiert als 1-Alkohole: bei niedrigen Temperaturen wird er mehr dehydriert, bei hohen mehr dehydratisiert.

Katalysator Pt/Chromosorb W wirkt schwächer, eben in reiner Wasserstoffatmosphäre wird auf ihm von den Sauerstoffverbindungen wenig Sauerstoff abgespaltet, noch weniger, wenn nur 5 % Wasserstoff anwesend ist. Die quantitativen Resultate der Experimente auf diesem Katalysator sind auf Fig. 2-II dargestellt. Wegen der Verlangsamung der Hydrogenolysereaktionen werden primäre und sekundäre Alkohole in hohem Masse dehydriert. Die Menge des von sekundärem Butanol abgespaltenen Wasserstoffs verändert sich, abhängig von der Temperatur, sehr wenig. Wahrscheinlich hängt diese von der Dehydratation bei höheren Temperaturen ab. Aus Fig. 2-II folgt es, das bei 250° aus 1-Alkoholen wenig Wasserstoff abgespaltet wird,

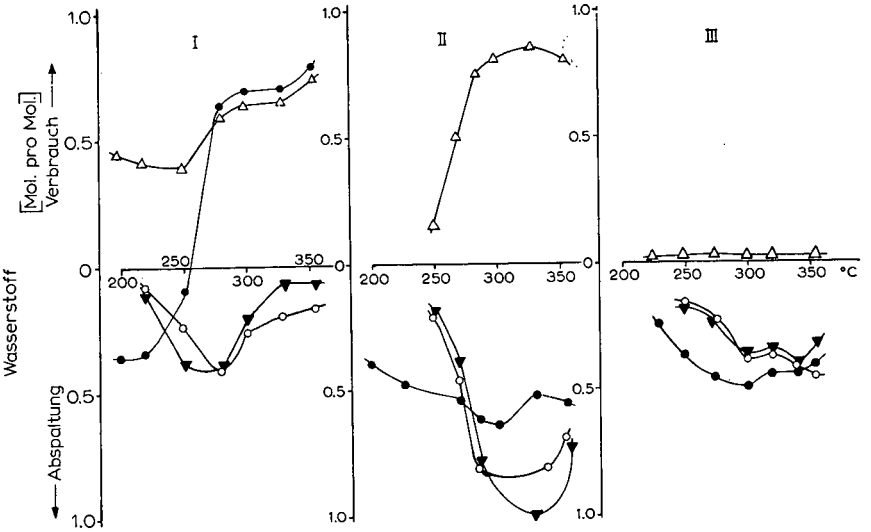


Fig. 2. Abspaltung und Verbrauch des Wasserstoffs von den isomeren Butanolen (Molekül pro Molekül) in Abhängigkeit von Katalysator und Katalysetemperatur. Katalysatoren: (I) Pd/Kieselguhr; (II) Pt/Chromosorb W; (III) Cu/Chromosorb W. Die Komponenten: (-○—○-) primärer *n*-Butylalkohol; (-●—●-) sekundärer *n*-Butylalkohol; (-▼—▼-) primärer Isobutylalkohol; (-△—△-) tertiärer Isobutylalkohol.

aber bei 300–320° zweimal mehr als aus sekundärem Butanol. Dieser Unterschied kann zur Feststellung ihrer Struktur dienen. So, wie der Wasserstoffverbrauch des tertiären Butanols es zeigt, wird er auch von diesem Katalysator mit guter Ausbeute dehydratisiert.

Ein Kupferkatalysator unterscheidet sich bedeutend von den anderen; auf ihm wird ein Olefin sehr wenig hydriert, auch der Wasserstoffbedarf des tertiären Butanols ist gering (Fig. 2-III). Dehydrierung anderer Butanolen hängt stark von der Temperatur ab. Sekundäres Butanol reagiert bei höheren Temperaturen ebenso wie 1-Alkohole, bei niedrigen wird es etwas leichter dehydriert.

Die beschriebenen Versuche wurden im Gasstrom Ar + 5% H_2 durchgeführt. Im reinem Argon können Hydrierungsreaktionen nicht ablaufen, deswegen wird der Berg aus tertiärem Butanol nicht erhalten. Die Dehydrierung von Alkoholen im Argonstrom verläuft mit etwas grösserer Ausbeute als beim Vorhandensein von Wasserstoff. Auf den Katalysatoren Pt/Chromosorb W und Kupfer erhielten wir fast gleiche Resultate, nur die Reaktionen von primären und sekundären Butanolen wiesen keine Unterschiede auf. Auf aktivem Katalysator Pd/Kieselguhr war bei 330–350° die relative Ausbeute von Wasserstoff aus sekundärem Butanol geringer, wahrscheinlich wegen der Dehydratation.

EINFLUSS DER MOLEKÜLGRÖSSE UND DOPPELBINDUNGEN

In der Literatur finden wir Angaben, dass hochmolekuläre primäre Alkohole und aromatische Alkohole bei Katalyse der Dehydratation leicht unterliegen[12], welcher Umstand die Ausbeute von Karbonylverbindungen verringert. Unsere Experimente mit *n*-Alkoholen (bis C_{10}) und Benzylalkohol vergewisserten, dass auf dem

aktivem Katalysator mit Vergrösserung des Molekülgewichts die Wasserstoffberge tatsächlich schnell sinken und Benzylalkohol Wasserstoff verbraucht. Auf passiven Katalysatoren (Pt/Chromosorb W und Cu) gab es solche Unterschiede nicht; hochmoleküläre Alkohole wurden ebenso dehydriert wie Butanole.

Es ist bekannt, dass konjugierte Doppelbindungen auf die Reaktionsfähigkeit gewisser funktioneller Gruppen einen Einfluss ausüben[13]. Wir haben *n*-Propylalkohol und Allylalkohol dehydriert. Es stellte sich dabei heraus, dass im Argonstrom auf Pt/Chromosorb W, bei 200–250°, Wasserstoffentwicklung mit Allylalkohol mehrmals grösser war, als mit Propylalkohol. Das bedeutet, dass der Wasserstoff zur gleichzeitigen Sättigung der olefinischen Bindung nicht gebraucht wurde, die Doppelbindung isomerisierte sich nicht. Bei preparativer Dehydrierung aber erhält man aus Allylalkohol meistens Propylaldehyd[12].

PRAKTISCHE ANWENDUNG

Bei Trockendestillation fester Brennstoffe entsteht auch Schwelwasser, das niedrigsiedende Heteroverbindungen in sich aufgelöst. In Fig. 3-I ist ein Chromato-

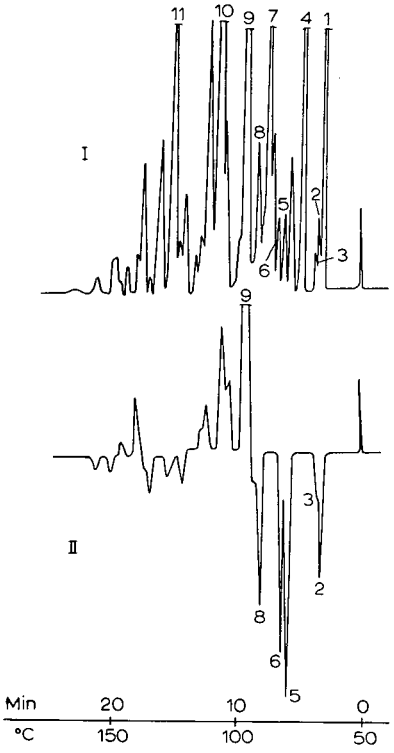


Fig. 3. Bestimmung der in kleiner Menge vorhandenen Alkoholen in Ketonen, die aus Brennschiefer-Schwelwasser entfernt wurden. (I) Chromatogramm des ursprünglichen Gemisches. (II) Ergebnisse der Katalyse bei 330° auf Pt/Chromosorb W. Programmierung der Säulentemperatur von 50° bis 200°. Identifizierte Verbindungen: (1) Methyläthylketon; (2) Isopropylalkohol; (3) Äthylalkohol; (4) Diäthylketon; (5) sekundärer *n*-Butylalkohol; (6) *n*-Propylalkohol; (7) Methylbutylketon; (8) primärer *n*-Butylalkohol; (9) Methylbutylketon mit Olefinbindung; (10) Cyclopentanon; (11) Cyclohexanon.

gramm der aus Brennschiefer-Schwelwasser entfernten Verbindungen dargestellt. Es sind hauptsächlich Ketone, die Alkohole sind in sehr kleiner Menge vorhanden. Zu ihrer Identifizierung wurde auf Pt/Chromosorb W, bei 330°, im Ar + H_2-Strom dehydriert. Die Ergebnisse sind in Fig. 3-II dargestellt. Es entstand auch ein positiver Berg, der gleich die Anwesenheit eines tertiären Alkohols oder einer ungesättigten Verbindung andeuten kann. Zur Feststellung des letzteren wurde das Gemisch in flüssiger Phase auf Raney-Nickel hydriert[14]. Bei der Dehydrierung Hydrogenisats erhielt man keinen positiven Berg mehr: folglich gehörte der fragliche Berg zu einer Doppelbindung.

Die Chromatogramme zeigen, dass man diese Methode auch zur Feststellung der in Spuren anwesenden Alkoholen anwenden kann.

DISKUSSION

Die erhaltenen Angaben zeigen, dass primäre Alkohole auf den beschriebenen Katalysatoren immer Wasserstoff abspalten, tertiäre aber werden dehydratisiert. Diese Eigenschaften können zu Unterscheidung derselben dienen. Schwieriger ist die Identifizierung sekundärer Alkohole, hier kann man zwei Erfahrungen ausnützen:

(1) Bei 300–320° auf aktivem Katalysator werden sekundäre Alkohole dehydratisiert (positive Berge), auf passivem Katalysator aber dehydriert (negative Berge).

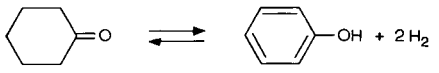
(2) Bei 250° werden sekundäre Alkohole leichter dehydriert als primäre.

Die olefinischen Bindungen und tertiären Hydroxylgruppen kann man mit vorsichtiger Hydrierung in flüssiger Phase unterscheiden. Dabei reagieren nur die ersten.

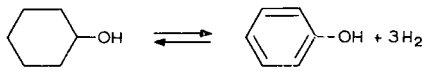
Alle beschriebenen Experimente wurden vergleichend in Argon + Wasserstoff und in reinem Argon ausgeführt. Dehydrierung im Argonstrom haben vor kurzem auch FRANC UND MIKÉS[11] zur gruppenmässigen Identifizierung mehrerer organischer Stoffe benützt. Unsere Erfahrungen haben gezeigt, dass dem Gasgemisch der Vorrang gegeben werden soll. In Anwesenheit des Wasserstoffs kann man Hydrierungs- und Hydrogenolysereaktionen durchführen. Die katalytische Wirksamkeit des Katalysators ist beständig und selektiv.

Zur Untersuchung von Alkoholen sind die angewandten aktiven und passiven Pt- und Pd-Katalysatoren, ihren katalytischen Eigenschaften gemäss benützt, gleich brauchbar. Auf dem Kupferkatalysator laufen Hydrogenationsreaktionen nicht ab, deswegen ist seine Benutzung für Dehydrierung von Alkoholen ziemlich begrenzt.

Bei der Katalyse in Anwesenheit Wasserstoffs geben andere Sauerstoffverbindungen, ausser den primären und sekundären Alkoholen, den Wasserstoff nicht ab. Man soll aber berücksichtigen, dass bei höheren Temperaturen alle sauerstoffenthaltenden Cyclohexanderivaten aromatisiert werden können und dabei Wasserstoff abgeben. So bestehen die Gleichgewichte[15]:

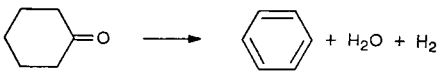


(4)



(5)

Falls Aromatisierung unter gleichzeitiger Hydrogenolyse Sauerstoffs erfolgt, bleibt noch Wasserstoff übrig:



(6)

Experimente auf aktivem Katalysator Pd/Kieselguhr ergaben, dass bei 300° Cyclohexanone, im Vergleich mit Alkoholen, sehr wenig Wasserstoff abgaben. Bei 350° aber waren Wasserstoffberge von Cyclohexanolen und Cyclohexanonen gleich gross, die von den aliphatischen Alkoholen dagegen niedrig. Von diesen Angaben ist zu ersehen, dass beide Gruppen von Cyclohexanderivaten mit gleicher Geschwindigkeit aromatisiert werden, und das erst bei höheren Temperaturen. Reichliche Abgabe Wasserstoffs bei 350° kann zur Erkennung von Cyclohexanstruktur in Sauerstoffverbindungen dienen.

ZUSAMMENFASSUNG

Die Alkohole wurden im Argonstrom, der 5 % Wasserstoff enthielt, getrennt und nachdem in einem der Säule nachgestellten Reaktor dehydriert. Dabei entstanden unter Wasserstoffabspaltung aus primären und sekundären Alkoholen Carbonylverbindungen. Die tertiären Alkohole wurden dehydratisiert und die so entstandenen Olefine gesättigt. Da die Komponenten dann vollständig adsorbiert wurden, registrierte der Detektor nur den entstandenen Wasserstoff und dessen Vakanzen, als negative und positive Berge.

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CHROM. 3664

MICRODETERMINATION OF ESTANE IN EXPLOSIVE MIXTURES

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SUMMARY

A microanalytical method based on pyrolytic gas chromatography is described for the determination of Estane 5740 X2 in explosive mixtures. The explosive, octahydro-1,3,5,7-tetranitro-s-tetrazine, is separated from Estane in chloroform solution, and an aliquot is taken as a sample. The polymer is converted to cyclopentanone by controlled pyrolysis for gas chromatographic analysis. The peak area of the cyclic ketone is measured and related to Estane concentration by means of a calibration curve. Several PBX and synthetic samples were successfully analyzed by this method.

INTRODUCTION

Plastic-bonded explosives (PBX's), which are usually mixtures of HMX (octahydro-1,3,5,7-tetranitro-s-tetrazine) or RDX (hexahydro-1,3,5-trinitro-s-triazine) with some thermoplastic binder, are an important class of contemporary explosives. Most such mixtures can be analyzed on a macro scale by relatively simple techniques, such as solvent extraction, and such techniques suffice for routine quality control. In research and development work, however, accurate analyses are frequently required on very small samples. In the present paper, we describe a procedure, based on pyrolytic gas chromatography (PGC), which is adaptable to the analysis of HMX–Estane mixtures on a semimicro scale.

Under controlled pyrolysis, Estane 5740 X2 (B. F. Goodrich) was found to yield a distinctive product, cyclopentanone, in an amount which was proportional to the Estane concentration in the sample. In PBX samples, however, the cyclopentanone is susceptible to oxidation by the pyrolysis products of HMX, which leads to low results. It was found that this effect could be minimized by selectively dissolving the Estane in chloroform and taking an aliquot of the chloroform solution for pyrolysis. PBX and synthetic samples were successfully analyzed by this procedure with the standard deviation on synthetic samples being 0.12 % or less.

EQUIPMENT AND REAGENTS

An Aerograph HY FI Model 600-D gas chromatograph, a Perkin-Elmer (PE) pyrolysis unit, and a Varian Model G-14 recorder were used.

A 6 ft. long × 1/8 in. O.D. (0.094 in. I.D.) stainless steel tube was packed with 25 % (by wt.) of Carbowax 20 M coated on 60/80 mesh, acid-washed Chromosorb W, using a Mathronic XL-300 column packer. The packed column was preconditioned at 125° with helium carrier gas before use.

Estane 5740 X2 (61.47 % C, 7.53 % H, 2.66 % N, and 28.30 % O by elemental analysis, empirical formula $C_{27}H_{39}NO_9$) was dissolved in A.R. grade chloroform and used as standard. The standard Estane solution was prepared as needed.

Ultrapure hydrogen in H-size cylinders, purchased from The Matheson Company, was employed as the carrier gas for the PGC unit.

Three 25-ml and three 50-ml volumetric flasks, two Hamilton microsyringes (10-μl capacity), and 12 microporcelain combustion boats (No. 00000) were needed for sample preparation.

An ultrasonic shaker was used to dissolve the PBX sample in chloroform. A centrifuge was used to separate the undissolved HMX granules.

EXPERIMENTAL

The tricresyl phosphate partitioning column, used to identify the major pyrolysis products of Estane[1], was unsuitable for quantitative work. Owing to excessive tailing of the cyclopentanone peak, the measurement of the peak area was difficult. A 6-ft. Carbowax column was found to separate the cyclic ketone as a symmetrical peak with a reasonable retention time from the other pyrolysis products. With this column, the pyrolysis and gas chromatographic conditions required to obtain a maximum conversion of Estane to cyclopentanone were determined.

Known amounts of Estane standards were pyrolyzed at different temperatures, and the pyrolysis products were separated on the Carbowax column. The peak area

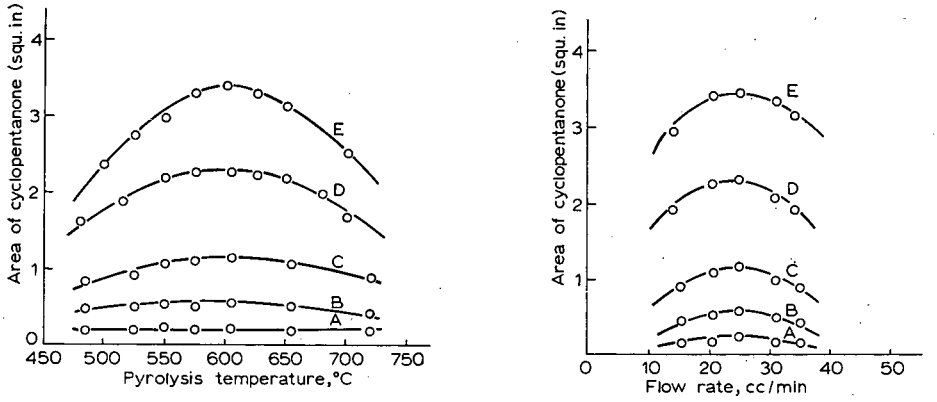

Fig. 1. Effect of pyrolysis temperature on Estane 5740 X2. Gas chromatographic conditions: Column, 6 ft. long × 1/8 in. O.D. (0.094 in. I.D.) 25% Carbowax 20 M on 60/80 mesh, acid-washed Chromosorb W; column temp., 85°; inlet temp., 100°; hydrogen carrier gas, flow rate 25 cc/min; recorder speed, 16 in./h; electrometer range, 1; attenuation × 4–× 16. Sample size: curve A, 2 μg Estane; curve B, 5 μg Estane; curve C, 10 μg Estane; curve D, 20 μg Estane; curve E, 30 μg Estane. Each point represents an average of duplicate runs.

Fig. 2. Effect of flow rate on Estane 5740 X2. Gas chromatographic and pyrolysis conditions: gas chromatographic conditions, same as Fig. 1, except variable flow rates; pyrolysis temp., 600°. Sample size: same as Fig. 1.

of cyclopentanone as a function of the pyrolysis temperature is shown graphically in Fig. 1. It can be seen that at 600° a maximum yield of cyclopentanone is obtained per given weight of polymer. At pyrolysis temperatures less than optimum, a measurable decrease in the cyclic ketone is noted.

With a pyrolysis temperature of 600° and the gas chromatographic conditions given in Fig. 1, the effect of the hydrogen flow rate on the cyclopentanone yield was determined. Fig. 2 shows that at between 24 and 25 cc/min of hydrogen the amount of cyclopentanone produced is a maximum.

It was anticipated that the pyrolysis of Estane in the presence of HMX would affect the yield of cyclopentanone. To establish the effect, a constant amount of Estane was mixed with varying amounts of HMX, and the mixed samples were subjected to pyrolysis. Fig. 3 shows that, with increasing amounts of HMX, the recovery of cyclopentanone decreases.

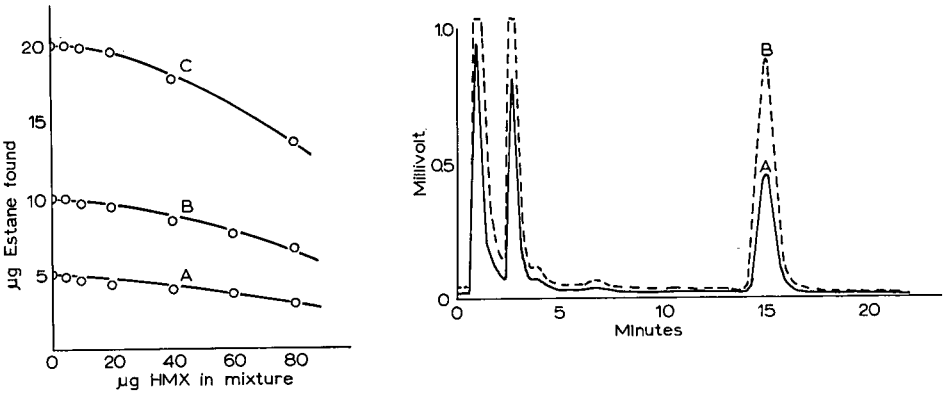

Fig. 3. Effect of mixed sample pyrolysis on Estane recovery. Chromatographic and pyrolysis conditions: column, 6 ft. long × 1/8 in. O.D. (0.094 in. I.D.), 25% Carbowax 20 M on 60/80 mesh, acid-washed Chromosorb W; column temp., 85°; inlet temp., 100°; hydrogen carrier gas, flow rate 24–25 cc/min; recorder speed, 16 in./h; electrometer range, 1; attenuation × 4–× 16; pyrolysis temp., 600°. Sample size: curve A, varying amounts of HMX mixed with 5 μg Estane 5740 X2; curve B, varying amounts of HMX mixed with 10 μg Estane 5740 X2; curve C, varying amounts of HMX mixed with 20 μg Estane 5740 X2. Each point on the curve represents an average value of two or more determinations.

Fig. 4. Pyrograms of Estane 5740 X2. Chromatographic and pyrolysis conditions: same as Fig. 3. Sample size: curve A (———), 5 μg Estane 5740 X2; curve B (-------), 10 μg Estane 5740 X2. Peaks A and B are cyclopentanone.

To eliminate the interference by HMX, chloroform was used as a selective solvent for the Estane. HMX is soluble to the extent of about 0.012 g per 100 ml of chloroform at 20°, while more than 0.200 g of Estane dissolves in the same amount of solvent. On the basis of these data, 1% PBX solutions and 10-μl aliquot samples were selected for use. Figs. 4 and 5 are pyrograms of Estane 5740 X2, HMX, and PBX 9011, an HMX/Estane mixture. With chloroform, only a negligible amount of HMX appears in the sample, and it causes no interference with the analysis.

Procedure

An accurately weighed 0.5-g sample of PBX is dispersed in 50 ml of chloroform with the aid of an ultrasonic shaker until no large clusters of PBX granules are visible.

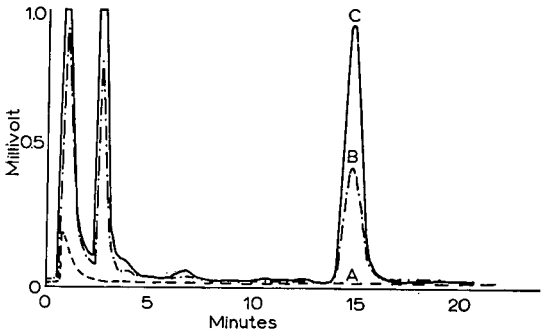

Fig. 5. Pyrograms of HMX, synthetic, and PBX 9011-06 samples. Chromatographic and pyrolysis conditions: same as Fig. 3. Sample size: curve A (-------), 10 μl $CHCl_3$ solution saturated with HMX; curve B (-·—·-), 10 μl 1 % synthetic sample (Estane–HMX, 5:95) = 5 μg Estane; curve C (———), 10 μl 1% PBX 9011-06 in $CHCl_3$ = 10 μg Estane. Peaks B and C are cyclopentanone.

The solution is centrifuged to separate the undissolved HMX crystals. A 10-μl aliquot is pipetted into a microprocelain combustion boat. Six ceramic boats with samples are loaded into the PE pyrolysis unit. The pyrolysis unit is closed and purged with the hydrogen carrier gas from the gas chromatograph at a flow rate of 24-25 cc/min as measured at the detector outlet. The column and the sample inlet temperatures are maintained at 85 and 100°, respectively. The recorder is turned on, and, after the electrometer is balanced, the flame ionization detector is ignited. The sample is moved with a magnet and a boat pusher into the pyrolysis chamber set at 600°. The pyrolysis products are swept into the gas chromatograph by the carrier gas and separated on the 6-ft. 25 % Carbowax column. The area of the cyclopentanone peak is measured by the peak height × peak width at half-height method and is related to Estane concentration by means of a calibration curve previously prepared with Estane standards.

RESULTS AND DISCUSSION

The procedure was applied to the analysis of several synthetic mixtures and PBX samples with the results shown in Table I. It is evident that the results are excellent. With synthetic mixtures, the standard deviation is about 0.1 %; PBX 9011 shows slightly higher values, but they are within the acceptable limits.

TABLE I

ANALYSIS OF ESTANE 5740 X2

Sample	*Nominal composition*	*No. of determinations*	*Percent estane found*	*S.D.*
Synthetic mix	HMX–Estane 5740 X2, 95:5	5	4.9	0.07
Synthetic mix	HMX–Estane 5740 X2, 90:10	5	9.8	0.12
PBX 9011-02	HMX–Estane 5740 X2, 90:10	10	9.8	0.18
PBX 9011-06	HMX–Estane 5740 X2, 90:10	10	9.8	0.27

The origin of cyclopentanone from the pyrolysis of Estane 5740 X2 was briefly investigated. Although the exact chemical composition of this polymer is not available, a closely related polymer, Estane 5740 X1, involves the condensation of 4,4'-diphenylmethane diisocyanate, 1,4-butanediol, and adipic acid[2]. If similar reactants are used in different proportions to prepare Estane 5740 X2, one of the reactants must be responsible for the formation of cyclopentanone. Each reactant was pyrolyzed in the same manner as the PBX samples, and adipic acid and its *n*-butyl ester produced the cyclic ketone as shown in Fig. 6. Therefore, the adipate group in Estane 5740 X2 yields the cyclopentanone product.

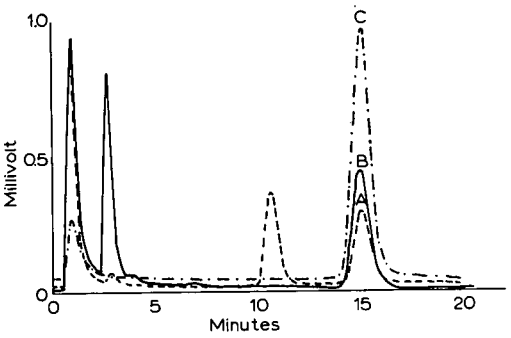

Fig. 6. Pyrograms of adipic acid, *n*-butyl adipate, and Estane 5740 X2. Chromatographic and pyrolysis conditions: same as Fig. 3. Sample size: curve A (-------), 20 μg *n*-butyl adipate, attenuated × 16; curve B (———), 5 μg Estane 5740 X2, attenuated × 4; curve C (–·—·–), 20 μg adipic acid, attenuated × 16. Peaks A, B, and C are cyclopentanone.

ACKNOWLEDGEMENT

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CHROM. 3666

GAS–LIQUID CHROMATOGRAPHY OF PROTEIN AMINO ACIDS

SEPARATION FACTORS*

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SUMMARY

This research reports on chromatographic and instrumental studies which will permit one to accurately, rapidly, and routinely analyze the twenty natural protein amino acids in biological substances. A number of factors influencing the performance of the chromatographic system were evaluated, and the separation characteristics of various polyesters of neopentyl glycol were evaluated. Also, an evaluation was made of support materials which had undergone various heat treatments.

It was shown that the optimum chromatographic performance for neopentyl glycol polyesters was observed at a carbon chain length of ten (neopentyl glycol sebacate). The separation ability of ethylene glycol adipate as a liquid phase was found to be superior to neopentyl glycol sebacate, consistent, and reproducible with respect to time and temperature. Arginine, histidine, and cystine were not reproducibly eluted from this column. This is a result of interaction between the substrate phase and the amino acid derivative. However, seventeen of the amino acid derivatives were well separated and quantitatively eluted in 33 min from columns containing 0.325 w/w % ethylene glycol adipate coated on 80/100 mesh acid-washed heat-treated Chromosorb G.

In general, the retention temperatures for the amino acids were lower and a significant improvement in resolution was noted when columns were prepared with Chromosorb G which had been heated at 450° to 600° for 15 h.

For the analysis of arginine, histidine and cystine, columns containing 1.5 w/w % OV-17 coated on high performance 80/100 mesh Chromosorb G were used. Di-acyl histidine was converted to the mono-acyl derivative by injection of *n*-butanol immediately after injection of the sample. The di-acyl derivative of histidine does not interfere with any of the amino acids on the EGA column.

A dual column chromatographic system of ethylene glycol adipate and OV-17 as the liquid phases is described which quantitatively separates the protein amino acids in 55 min. The quantitative gas–liquid chromatographic analysis of the amino acids in ribonuclease is reported.

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** Experimental data taken in part from master's thesis, University of Missouri, June, 1968.

*** Experimental data taken in part from master's thesis, University of Missouri, February, 1968.

INTRODUCTION

Gas–liquid chromatographic (GLC) methods have been particularly useful for the rapid, accurate, and sensitive analysis of biological materials for their amino acid content. However, this technique has presented certain limitations concerning the routine determination of all twenty of the protein amino acids. The reaction conditions for quantitatively preparing the derivatives of the twenty protein amino acids and at least thirty non-protein amino acids have been determined[1–5]. The major remaining problem has been the inability to elute quantitatively and separate all of the derivatives from a single chromatographic column.

A general review by GEHRKE AND STALLING in 1967[1] discussed the preparation of suitable derivatives of amino acids and their analysis by GLC. LAMKIN AND GEHRKE[2] reported in 1965 that the most suitable derivative for the gas–liquid chromatography of the natural protein amino acids is the N-trifluoroacetyl (N-TFA) *n*-butyl ester. In the same year they reported a procedure for quantitatively preparing the N-TFA *n*-butyl esters of eighteen of the amino acids for GLC analysis. Single chromatographic peaks were obtained for all the common protein amino acids except tryptophan and arginine. Reproducibility of response was found to be good for derivatives carried through the entire chemical and chromatographic procedures.

Studies on the quantitative gas chromatography of amino acids were reported by GEHRKE *et al.*[3] in 1965. The retention temperatures and relative molar responses of the amino acids were determined using a 1.0 m column packed with 1.0 w/w % neopentyl glycol succinate on 60/80 mesh Gas-Chrom A. Acylation studies on arginine were published in 1966 by STALLING AND GEHRKE[4]. The quantitative conversion of arginine to a single derivative, the tris(trifluoroacetyl) derivative, was achieved by conducting the acylation of arginine *n*-butyl ester·HCl at 150° for 5 min in a *closed* tube. Also, tryptophan was converted to the di-acyl derivative producing only one peak, and identical relative peak areas were obtained for each of the other eighteen protein amino acids using either the closed tube acylation method at 150° for 5 min or acylation at room temperature for 2 h.

An investigation of chromatographic factors affecting the separation of the amino acid N-TFA *n*-butyl esters was reported by GEHRKE AND SHAHROKHI[5] in 1966. The aim of that study was to find suitable column packings which would completely separate and allow the quantitative elution of the twenty volatile derivatives on a single column. An evaluation was made of a number of the commercially available polar stationary phases. Combinations of two mixed stationary phases were investigated as well as columns packed with two liquid phases in series. This paper also reported on different column lengths and concentrations of the two stationary phases and the use of temperature programming. Complete resolution of a 20-component mixture of the N-TFA *n*-butyl ester derivatives was achieved with a *mixed* stationary phase column of 0.75/0.25 w/w % of DEGS/EGSS-X. However, reproducible elution of arginine, histidine, and cystine was not obtained using this column. Also, this mixture of stationary phases was found inadequate for routine analysis of the amino acids in proteins as isoleucine and glycine were unresolvable after prolonged use of the column. STEFANOVIC AND WALKER[6] reported that the liquid phase ethylene glycol adipate (EGA) would separate the twenty N-TFA *n*-butyl ester derivatives. These workers reported the optimum loading to be 0.65 w/w % EGA on Chromosorb

W. However, they observed very small peaks for arginine, histidine and cystine, and suggested that these derivatives were decomposed on the column or reacted chemically with the polar liquid phase.

McBRIDE AND KLINGMAN[7] investigated polyesters of increased thermal stability in an effort to find a single column which would separate nanomolar quantities of the N-TFA *n*-butyl esters of amino acids isolated from biological substances. Using a 1.2 w/w % phenyldiethanolamine succinate polyester coated on Gas-Chrom A, they reported good separation for seventeen amino acids but gave no data for arginine, histidine and cystine. DARBRE, BLAU and ISLAM[8,9] have investigated the derivatization and GLC separation of the amino acids. They made extensive use of mixed siloxane liquid phases for the separation of the N-trifluoroacetyl methyl esters. However, histidine was not quantitatively eluted from these columns.

The present paper reports research on chromatographic and instrumental studies which will permit one to analyze the protein amino acids accurately, rapidly, and routinely in biological substances. In this work, a number of factors influencing the performance of the chromatographic system were evaluated, the results being a dual column system which completely resolves and quantitatively elutes in a routine manner the twenty N-TFA *n*-butyl ester derivatives.

Further, in an effort to find a stationary phase which would resolve all of the N-TFA *n*-butyl esters of the protein amino acids, the separation characteristics of various polyesters of neopentyl glycol were evaluated. The effect of increasing carbon chain length of the dicarboxylic acids in the polyesters on the retention volumes of the amino acid derivatives was studied.

Also, an evaluation was made of support materials which had undergone various heat treatments to determine the effect of removal of moisture from the support phase prior to coating with the substrate phase.

Investigations were made on a dual column system containing 0.325 w/w % EGA polyester liquid phase for the separation of seventeen of the amino acid derivatives and 1.5 w/w % OV-17 siloxane liquid phase for the elution of arginine, histidine and cystine. To improve the quantitation of histidine it was converted from the di-acyl to the mono-acyl derivative.

EXPERIMENTAL

Apparatus

A dual hydrogen flame detector gas chromatograph (Microtek Model 220), with strip chart recorders (Texas Instruments, Inc., Model FS1N6A), and a digital readout integrator (Infotronics, Model CRS-11AB/HS/42) were used. A Varian Aerograph Model 1520 dual column unit with two flame ionization detectors and matrix temperature programming was also utilized. Also, a Varian Aerograph Model 2100 instrument with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and linear temperature programming was used. Both Varian units were equipped with Leeds and Northrup Speedomax W recorders with Model 224 Disc Chart Integrators.

Solvents were removed from samples and reaction mixtures by means of a CaLab rotary evaporator, "Cold finger" condenser, and a Welch Duo-Seal vacuum pump. An electric furnace (Arthur H. Thomas, Co.), vacuum oven and vacuum

desiccator (Precision Scientific Co.) were used to remove moisture from the support material.

Reagents

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc. or Nutritional Biochemicals Corp., and were chromatographically pure.

Methanol and butanol were "Baker Analyzed" reagents. Chloroform was a "Fisher Certified Reagent". The trifluoroacetic anhydride was obtained from Distillation Products Industries, and was an "Eastman Grade" chemical. Acetonitrile was a "Baker Analyzed" reagent of "Nanograde" purity. Anhydrous HCl, 99.0 % minimum purity, was obtained from Matheson Company. *p*-Toluenesulfonic acid was purchased from K and K Laboratories.

The methanol and methylene chloride were redistilled from an all glass system protected from atmospheric moisture. The methanol was first refluxed over magnesium turnings, and the methylene chloride over calcium chloride before distillation. The anhydrous HCl gas was passed through a H_2SO_4 drying tower before bubbling through the butanol or methanol.

Column packings

The following materials were purchased from Analabs, Inc.: neopentyl glycol succinate (NPGS), neopentyl glycol adipate (NPGA), neopentyl glycol sebacate (NPGSb), and ethylene glycol adipate (EGA). The OV-17 substrate was obtained from Applied Science Laboratories, Inc., and DC-550 was purchased from Dow Corning Corp. Neopentyl glycol and brassylic acid were obtained from K and K Laboratories, Inc.

Neopentyl glycol brassylate (NPGB) was prepared as described by JAMES[10], using *p*-toluenesulfonic acid as esterification catalyst. The product was a viscous liquid which solidified at room temperature.

The support material, 80/100 mesh acid-washed Chromosorb G, was obtained from Johns-Manville. 30.000 g of this material were weighed into each of five quartz evaporating dishes, and heated as described prior to coating with the liquid phase. Five different heat treatments were made: (a) 15 h at 100° in a vacuum oven, and (b, c, d and e) all for 15 h in a muffle furnace at 300°, 450°, 600° and 850°, respectively.

Then the dried support phases were allowed to cool in a vacuum desiccator over P_2O_5, and weighed to determine weight loss.

Columns were then prepared using each of the heated supports to observe any change in column performance as a function of the heat treatment. The liquid phases used were 0.5 w/w % NPGSb and 0.325 % w/w EGA.

Column preparation

A known amount of support was added to a 500 ml ridged round bottom flask and covered with the solvent used to dissolve the liquid phase. The stationary phase was weighed into a small Erlenmeyer flask, dissolved in the appropriate solvent, and transferred to the flask containing the support. The flask containing the support and stationary phase was then placed in a 60° water bath, and the solvent was slowly removed with a rotary evaporator and vacuum pump. Glass columns 1.5 m × 4 mm

I.D. were packed by slow addition of the coated support with gentle tapping of the column. Dry glass wool plugs ($^1/_4$ in.) were then placed in the ends of the column.

Four columns containing 0.5 w/w % NPGS, NPGA, NPGSb, and NPGB were placed in the gas chromatograph and conditioned simultaneously. The support material in each case was 80/100 mesh acid-washed Chromosorb G. These columns were conditioned for 15 h at 220° at a carrier gas flow rate of 10 ml/min N_2. Also a 0.325 w/w % EGA column was prepared and packed in a 1.5 × 4 mm I.D. glass column.

Instrumental settings

Column temperature	initial, 65°; final, 220°
Detector temperature	260°
Program rate	2°/min
N_2 carrier flow	64 ml/min
Hydrogen	35 ml/min
Air	400 ml/min
Chart speed	0.25 in./min

Preparation of derivatives

To evaluate the separation characteristics of the liquid phases NPGS, NPGA, NPGSb, and NPGB, a mixture of eight amino acids was converted to their N-TFA *n*-butyl derivatives as described by GEHRKE *et al.*[2]. This mixture included alanine, valine, glycine, isoleucine, leucine, threonine, proline, and serine. These amino acid derivatives are the eight of greatest volatility. The same procedure was used to prepare derivatives for the study of EGA as a liquid phase, and for the evaluation of the heated support materials.

RESULTS AND DISCUSSION

Substrate phase studies

Earlier experiments by GEHRKE AND SHAHROKHI[5] with the previously mentioned mixed liquid phases showed that they were unsuited for routine analysis as the separation and elution characteristics of the column varied with time and temperature. Thus, neopentyl glycol polyesters of increasing carbon chain length were evaluated to determine their ability to resolve the N-TFA *n*-butyl esters of the protein amino acids. The retention temperature of the eight amino acid derivatives on each neopentyl glycol polyester column was noted under similar chromatographic conditions. Table I gives the relative retention volumes for these amino acid derivatives. Alanine was assigned an arbitrary value of 1.00. Fig. 1 graphically shows the relative retention volume *versus* the carbon number of the dicarboxylic acid. From this graph it is clear that maximum separation was achieved at carbon number 10, or neopentyl glycol sebacate. From a structural standpoint there are ten atoms from the trifluoromethyl group to the terminal methyl group of the butyl ester of the amino acids. Also, in the case of leucine and isoleucine there are ten atoms including the terminal aliphatic methyl group to the terminal carbon atom in the *n*-butyl group. Maximum separation could result from the preferential orientation of the aliphatic portion of the amino acid with the CH_2 groups of the polyester and of the polar trifluoroacetyl group with the ester groups in the polyester.

TABLE I

SEPARATION OF AMINO ACIDS WITH POLYESTERS OF NEOPENTYL GLYCOL

Amino acid	*Relative retention volume*			
	Succinate	*Adipate*	*Sebacate*	*Brassylate*
Alanine (1.00)	1.00 (26.8)[a]	1.00 (27.7)	1.00 (25.6)	1.00 (21.1)
Valine	1.11 (29.8)	1.10 (30.4)	1.20 (30.8)	1.19 (25.2)
Isoleucine	1.29 (34.7)	1.29 (35.8)	1.46 (37.4)	1.25 (26.4)
Glycine	1.29 (34.7)	1.29 (35.8)	1.54 (39.5)	1.42 (29.9)
Leucine	1.48 (38.2)	1.40 (38.9)	1.66 (42.5)	1.56 (33.0)
Threonine	1.52 (40.7)	1.40 (38.9)	1.80 (46.5)	1.56 (33.0)
Proline	1.56 (42.0)	1.53 (42.5)	1.99 (51.0)	1.64 (34.3)
Serine	1.75 (46.8)	1.67 (46.2)	2.16 (55.3)	1.66 (35.0)

[a] Retention volume per mg liquid phase, ml/mg.

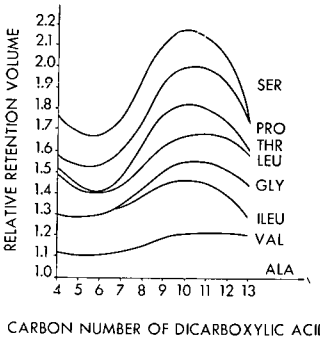

Fig. 1. Relative retention volumes of N-TFA *n*-butyl esters of amino acids with polyesters of neopentyl glycol.

The chromatographic resolution seen in Fig. 2 was achieved on further study with the NPGSb stationary phase. This liquid phase exhibited very good separation ability for the twenty protein amino acids under the appropriate instrumental conditions. However, continued investigation showed that this liquid phase was not satisfactory for the analysis of arginine, histidine and cystine as these derivatives

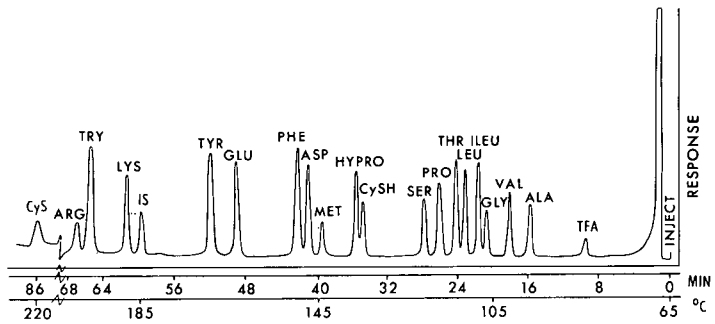

Fig. 2. Separation of amino acids with 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Program rate: 2°/min. Internal standard: *n*-butyl stearate. Concentration: 12.5 n*M* each.

were not quantitatively eluted. In many instances only diminished peaks were observed for these amino acid derivatives. However, NPGSb was found to be a good stationary phase for the analysis of seventeen of the protein amino acids.

Support phase studies

Since the N-TFA *n*-butyl ester derivatives of the amino acids are especially susceptible to hydrolysis, it was considered that any water which could be removed from the support material should improve the efficiency of a particular column and result in less support–derivative interaction. The loss of weight on heating the 80/100 mesh acid-washed Chromosorb G to various temperatures was observed. The thermal treatments used and the corresponding weight losses are given in Table II.

TABLE II

THERMAL TREATMENTS AND WEIGHT LOSSES OF CHROMOSORB G

Experiment	*Heat treatment*	*Weight loss* (%)
A	15 h, 100°, vacuum oven	0.08
B	15 h, 300°, muffle furnace	0.52
C	15 h, 450°, muffle furnace	0.54
D	15 h, 600°, muffle furnace	0.63
E	15 h, 850°, muffle furnace	0.64

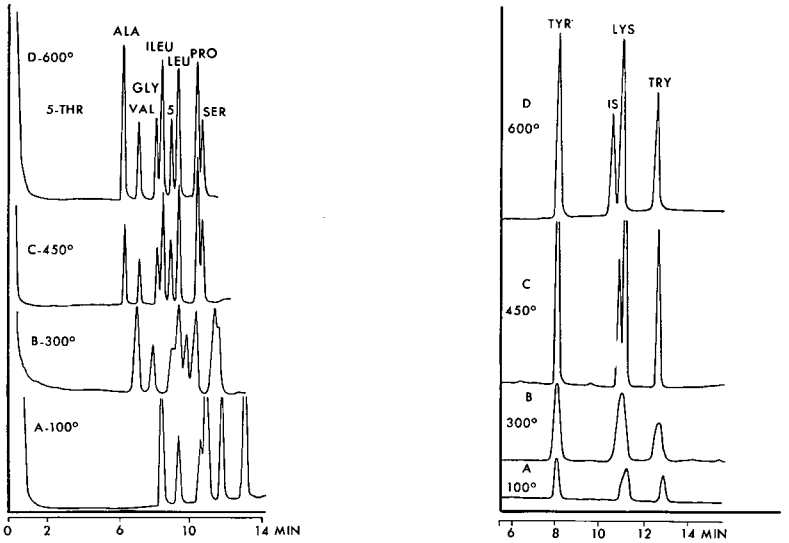

Fig. 3. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.0 m × 4 mm I.D., glass. N_2 flow: 70 ml/min. Temperature: 80° initial, 5°/min.

Fig. 4. Effect of heating of the inert support on resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.0 m × 4 mm I.D., glass. N_2 flow: 70 ml/min. Temperature: 150° initial, 5°/min.

The performance of these heat-treated (H.T.) support materials was observed after coating each with 0.5 w/w % of NPGSb. These columns were conditioned simultaneously under the same conditions and all of the instrumental settings were the same for each analysis. Fig. 3 shows a comparison of chromatograms obtained on the analysis of a mixture of the eight most volatile amino acid derivatives on support phases heated to different temperatures. An increase in separation was observed for the columns whose support had been heated in the range of 450–600°. Fig. 4 shows a similar comparison of heated supports for the derivatives of tyrosine, lysine, and tryptophan, with *n*-butyl stearate as the internal standard. Increased separation was again achieved with supports heated at 450–600°. A comparison of these heated support materials was then made using a mixture of seventeen amino acid derivatives (Fig. 5). In this experiment EGA was used as the liquid phase. The peak for tyrosine

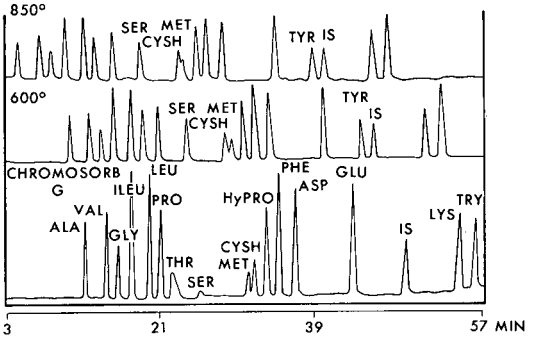

Fig. 5. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.325 w/w % ethylene glycol adipate (EGA) on 80/100 mesh acid-washed H.T. Chromsorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Temperature: 65° initial, 2°/min. Final temperature 225°.

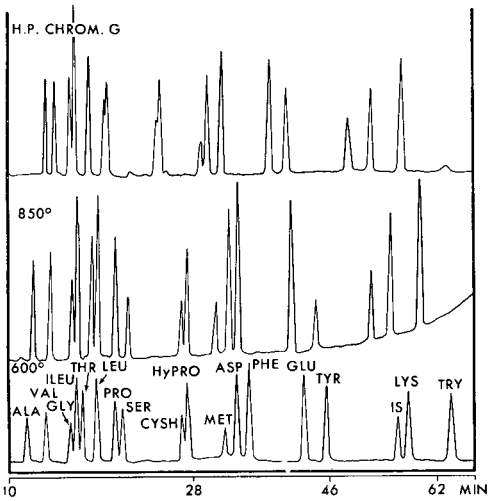

Fig. 6. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Temperature: 65° initial, 2°/min. Final temperature 240°.

was absent, and small for serine on regular Chromosorb G. For the non-heated support, tyrosine was partially converted to the free hydroxy derivative, and eluted with lysine. Fig. 6 shows a similar study with NPGSb as the liquid phase.

Some studies were also made with high performance Chromosorb G, a product silanized by the manufacturer. The retention temperature for each of the amino acid derivatives was noted for each column and is given in Tables III and IV. In general, a decrease in the retention temperature of the amino acids was noted for columns containing the heat-treated support.

From these studies, EGA was found to be the best liquid phase, and Chromosorb G heated at 600° for 15 h the best support phase, for the analysis of seventeen of the protein amino acids.

TABLE III

RETENTION TEMPERATURES OF N-TFA *n*-BUTYL AMINO ACID ESTERS ON CHROMOSORB G

Amino acid	*EGA 0.325 w/w % and Chromosorb G*			
	Acid-washed Chromosorb G	*Acid-washed Chromosorb G 500°*	*Acid-washed Chromosorb G 850°*	*H.P. Chromosorb G DMCS*[a]
Alanine	96	92	87	75
Valine	102	97	92	77
Glycine	105	101	94	81
Isoleucine	107	104	97	83
Leucine	112	108	102	84
Proline	115	111	104	93
Threonine	118	115	107	105
Serine	125	121	114	109
Cysteine	137	131	124	119
Methionine	139	133	125	121
Hydroxyproline	142	136	127	125
Phenylalanine	144	140	130	139
Aspartic acid	149	143	134	145
Glutamic acid	163	157	146	174
Tyrosine	175	165	155	176
Lysine	186	179	165	200
Tryptophan	190	183	168	219

[a] DMCS = dimethylchlorosilane.

The dual column chromatographic system

A chromatographic column of 0.325 w/w % ethylene glycol adipate (EGA) coated on 80/100 mesh, a.w., Chromosorb G previously heated to 600° for 15 h was selected for the separation of all the protein amino acids except arginine, histidine and cystine.

A 1.5 m × 4 mm I.D. U-shaped glass column containing this substrate and support phase was placed into the Model 2100 Aerograph gas chromatograph. A standard reference solution of the amino acid derivatives was prepared by taking a 10 ml aliquot of a solution containing 2.5 mmoles/l of each amino acid through the derivatization method. The closed tube acylation method was used. Five microliters of the derivatized solution were injected into the gas chromatograph.

For the determination of arginine, histidine, and cystine, columns of DC-550

TABLE IV

RETENTION TEMPERATURES OF N-TFA-*n*-BUTYL AMINO ACID ESTERS ON CHROMOSORB G

Amino acid	*NPGSb 0.5% w/w and Chromosorb G*			
	Acid-washed Chromosorb G	*Acid-washed Chromosorb G 500°*	*Acid-washed Chromosorb G 850°*	*H.P. Chromosorb G DMCS*
Alanine	107	97	99	99
Valine	109	103	104	102
Glycine	114	110	110	106
Isoleucine	115	112	112	107
Leucine	116	114	116	107
Threonine	118	118	119	112
Proline	123	122	123	116
Serine	123	125	126	117
Cysteine	137	140	138	130
Hydroxyproline	139	142	139	131
Methionine	148	151	147	142
Aspartic acid	151	154	150	143
Phenylalanine	153	158	153	147
Glutamic acid	167	170	167	158
Tyrosine	173	175	174	163
Butyl stearate	188	190	185	178
Lysine	192	193	190	183
Tryptophan	200	206	198	192
Arginine	—	209	—	—
Cystine	—	220	234	204

and OV-17 coated on 80–100 mesh, high performance (H.P.) Chromosorb G were prepared. These columns were placed in the Model 2100 Aerograph gas chromatograph and evaluated. This experiment resulted in the discovery of two peaks for histidine, *viz.* the mono-acyl and di-acyl derivatives. Then, mixtures of glutamic acid, arginine, histidine, and cystine were derivatized and chromatographed on columns of DC-550 and OV-17. Using extreme care to maintain the anhydrous condition of the sample it was possible to obtain only the di-acyl peak for histidine. This was the moderately volatile component with a retention temperature slightly lower than glutamic acid (top chromatogram, Fig. 7). The di-acyl derivative could be obtained from samples acylated in a closed tube or at room temperature. However, injection of samples that had been opened to the atmosphere a few times resulted in the two peaks for histidine (middle chromatogram, Fig. 7).

Chromatography of the samples on polyester substrate columns under more or less anhydrous conditions resulted in only the less volatile mono-acyl component and at a reduced level of response. It was assumed that the component of higher volatility was the N,N′-bis(trifluoroacetyl) derivative, and that an interaction with the polyester column material resulted in the conversion of the di-acyl histidine derivative to the mono-acyl product.

In addition to the extreme sensitivity of the histidine di-acyl derivative to hydrolysis, it was found that the separation of the di-acyl derivative from aspartic acid was impossible on the siloxane polymer substrate columns. Thus, for the analysis of histidine experiments were made to quantitatively convert the di-acyl derivative

of histidine to its mono-acyl derivative. This was accomplished by injecting 1-butanol into the gas chromatograph immediately after injection of the sample containing histidine (bottom chromatogram, Fig. 7).

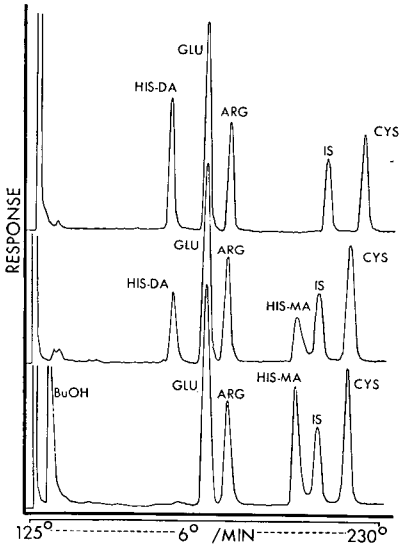

Fig. 7. Chromatograms of a solution containing histidine. Top: Anhydrous condition of sample maintained. Middle: Sample allowed contact with atm. H_2O. Bottom: BuOH injected on column after sample injection. Column: 1 m × 4 mm I.D., 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G. MA = Mono-acyl, DA = di-acyl.

RESULTS AND DISCUSSION (DUAL COLUMN SYSTEM)

Excellent separation of seventeen protein amino acids was obtained with the 0.325 w/w % EGA column. The instrumental operating and chromatographic conditions were as follows:

Column temperature	initial, 80°; final, 215°
Detector temperature	250°
Program rate	4°/min
N_2 carrier flow	60 ml/min
Hydrogen	50 ml/min
Air	450 ml/min
Chart speed	0.25 in./min

The evaluation of DC-550 and OV-17 as possible substrates for the analysis of arginine, histidine and cystine showed that either of these liquid phases coated on H.P. Chromosorb G would provide good separation. However, considerable substrate bleed was encountered with the DC-550 liquid phase at the elevated temperatures necessary to elute these amino acids of low volatility. Therefore, the OV-17 substrate on 80/100 mesh H.P. Chromosorb G was selected as the second column for the dual column system. A loading level of 1.5 w/w % of the OV-17 produced the most reproducible response factors for these amino acids while maintaining excellent separation.

At a higher loading level of 2.5 w/w %, cystine was not completely eluted from the column.

With a 1 m column of 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G in the Aerograph Model 1520 chromatograph it was found that the injection of 1 μl of 1-butanol immediately following the injection of the sample would convert all of the histidine to its mono-acyl derivative. One microliter of butanol was selected since it gave reproducible response factors for arginine and cystine as well as histidine when mixtures of the amino acids were analyzed. In order to achieve this conversion of histidine it was necessary to inject 7 μl 1-butanol onto the column when an Aerograph 2100 instrument was employed. The relative molar responses of arginine, histidine and cystine with respect to glutamic acid are presented in Table V. The instrumental conditions were as follows:

Column temperature	initial, 125°; final, 235°
Detector temperature	250°
Program rate	6°/min
Injection port temperature	200°
N_2 carrier flow	70 ml/min
Hydrogen	50 ml/min
Air	450 ml/min
Chart speed	0.5 in./min

TABLE V

RELATIVE MOLAR RESPONSE OF ARGININE, HISTIDINE AND CYSTINE ON DIFFERENT COLUMNS AND INSTRUMENTS

Amino acid	*Column and instrument*		$RMR_{a.a./glu.}$			*Average*
			1[a]	2	3	
Arginine	Old column[b],	Model 1520[c]	0.61	0.62	0.64	0.62
	New column[d],	Model 1520	0.70	0.69	0.68	0.69
	New column,	Model 2100[e]	0.76	0.74	0.74	0.75
Histidine	Old column,	Model 1520	0.37	0.37	0.35	0.36
	New column,	Model 1520	0.47	0.47	0.47	0.47
	New column,	Model 2100	0.49	0.46	0.47	0.47
Cystine	Old column,	Model 1520	0.94	0.95	0.88	0.92
	New column,	Model 1520	0.94	0.93	0.93	0.93
	New column,	Model 2100	0.62	0.63	0.60	0.62

[a] 1, 2, and 3 are independent samples.
[b] Extensively used column.
[c] Model 1520 Aerograph Gas Chromatograph. Samples injected in this instrument were followed by a 1 μl butanol injection.
[d] Well conditioned new column.
[e] Model 2100 Aerograph Gas Chromatograph. Samples injected in this instrument were followed by a 7 μl butanol injection.

A comparison of the relative molar responses obtained from different columns of the same material showed that it was essential that the response factors of the amino acid derivatives be determined periodically for that column (Table V). Columns of different ages and prior use will give different response factors. However, a column

can be used for at least 50 to 100 analyses. The relative molar response (RMR) of glutamic acid was arbitrarily assigned a value of unity. The relative molar response of each amino acid relative to glutamic acid, $RMR_{a.a./glu.}$, was calculated as follows:

$$RMR_{a\ a./glu.} = \frac{A_{a.a.}}{\text{moles}_{a.a}} \Big/ \frac{A_{glu.}}{\text{moles}_{glu.}}$$

where $A_{a.a.}$ = area in counts of amino acid peak.

A chromatogram of the complete separation of the twenty common protein amino acids with a dual column system is shown in Fig. 8. A complete gas chromatographic analysis of a protein hydrolysate can be made as follows:

Place a 1.5 m column of 0.325 w/w % EGA on 80/100 mesh acid-washed H.T. Chromosorb G in an instrument designed to accommodate at least two columns. Also place in the instrument a 1.0 m column of 1.5 w/w % of OV-17 on 80/100 mesh acid-washed H.P. Chromosorb G. By analyzing a derivatized reference standard solution on both columns, the relative molar response of each of the amino acids to

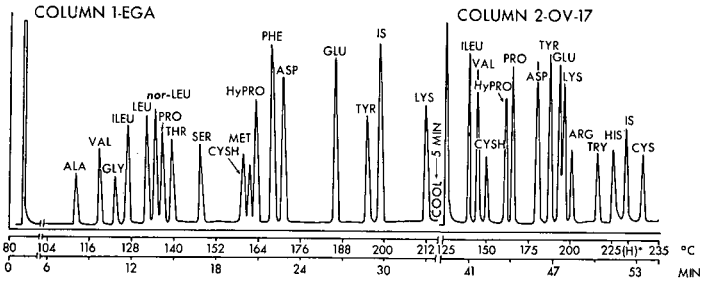

Fig. 8. Separation of all twenty protein amino acids with a dual column system. Column 1: 0.325 w/w % ethylene glycol adipate on 80/100 mesh acid-washed H.T. Chromosorb G, 1.5 m × 4 mm I.D., glass. Initial temperature: 80°, 4°/min. N_2 flow: 60 ml/min. Column 2: 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D., glass. Initial temperature: 125°, 6°/min. N_2 flow: 70 ml/min. The injected mixture contained *ca.* 0.02 μmole of each amino acid (~ 2 μg). *Hold at 235°.

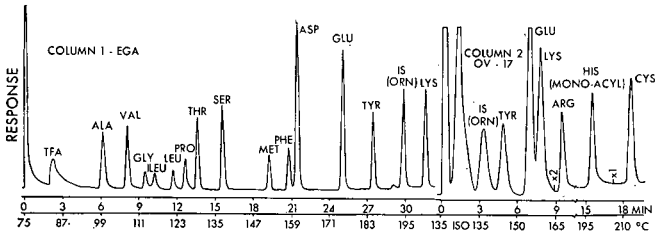

Fig. 9. Gas–liquid chromatogram of ribonuclease N-TFA *n*-butyl esters. Sample: 15 mg in 5.0 ml; 15 μg total amino acids injected (5 μl). Varian Aerograph 2100. EGA column: 0.325 w/w % on 80/100 mesh acid-washed H.T. Chromosorb G, 1.5 m × 4 mm I.D., glass. Initial temperature: 75°, 4°/min. OV-17 column: 1.5 w/w % on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D., glass. Initial temperature: 135°, 5°/min in Microtek MT 220. Three microliters (9 μg total) of sample injected followed by 5 μl *n*-butanol.

the internal standard can be calculated for the particular instrument and column employed. With these RMR factors determined, the protein sample hydrolysates after derivatization can be analyzed on both columns and the w/w % of each amino acid calculated.

The applicability of this method is demonstrated by the GLC analysis of ribonuclease. The complete chromatogram is shown in Fig. 9. Table VI presents the data obtained from both GLC and ion-exchange analyses of ribonuclease, and excellent agreement of the two was obtained.

TABLE VI

AMINO ACID ANALYSIS OF RIBONUCLEASE[a]

Amino acid	*w/w* %			
	Gas–liquid chromatography		*Average*	*Ion exchange chromatography*[b]
Alanine	7.27	7.47	7.37	7.60
Valine	6.81	7.08	6.95	7.00
Glycine	1.75	1.62	1.69	1.83
Isoleucine	1.67	1.61	1.64	2.06
Leucine	2.14	1.92	2.02	2.22
Proline	3.15	2.90	2.98	3.11
Threonine	7.78	7.94	7.86	7.80
Serine	9.66	9.81	9.74	9.41
Methionine	2.55	2.71	2.63	3.38
Phenylalanine	3.26	3.24	3.25	3.62
Aspartic acid	13.78	13.68	13.73	14.07
Glutamic acid	11.93	12.19	12.06	12.08
Tyrosine	6.43	6.29	6.36	6.80
Lysine	10.25	10.17	10.21	10.58
Arginine	5.04	4.83	4.93	4.65
Histidine	3.21	3.58	3.40	3.46
Cystine[c]	6.10	6.36	6.23	6.20
			103.05	105.87

[a] All determinations made on the same hydrolysate which was prepared by hydrolyzing the protein for 18 h at 105° in a closed tube with constant-boiling HCl. Ornithine used as the internal standard.

[b] Average of two independent samples, norleucine as internal standard.

[c] Analyzed as half-cystine by the ion-exchange method.

CONCLUSIONS

These studies have shown that by varying the length of the carbon chain in a polyester substrate phase, the separation characteristics of a chromatographic column can be markedly influenced. The optimum chromatographic performance for neopentyl glycol ployesters was observed at a carbon chain length of ten (neopentyl glycol sebacate).

Although NPGSb exhibited good separation ability of the N-TFA *n*-butyl esters of the protein amino acids, this polyester was not suited for the analysis of arginine, histidine and cystine, as these derivatives were not quantitatively eluted from the column. The separation ability of ethylene glycol adipate as a liquid phase

was found to be superior to NPGSb, consistent, and reproducible with respect to time and temperature. Again, arginine, histidine and cystine were not reproducibly eluted from this column.

The effects of heating the support material prior to coating with the stationary phase were pronounced. In general, the retention temperatures were lower and a significant improvement in resolution was noted when columns were prepared with Chromosorb G which had been heated at 450° to 600° for 15 h. Also, losses in weight (*ca.* 0.5 %) were observed on heating the support material, and the resultant loss of water improved the reproducible elution and separation of the amino acid derivatives when using polyester stationary phases. With unheated Chromosorb G, however, the peak for tyrosine was gone and only a small peak was observed for serine. This was corrected with the heat-treated Chromosorb G.

In all of our experiments it was not possible to elute quantitatively arginine, histidine and cystine from a polyester liquid phase. This is a result of interaction between the substrate phase and the amino acid derivative. However, seventeen of the protein amino acid derivatives were well separated and quantitatively eluted in 33 min from columns containing 0.325 w/w % EGA coated on 80/100 mesh acid-washed heat-treated Chromosorb G.

For the analysis of arginine, histidine and cystine, columns containing 1.5 w/w % OV-17 coated on high performance 80/100 mesh Chromosorb G gave excellent results. Di-acyl histidine was converted to the mono-acyl derivative by injection of *n*-butanol immediately after injection of the sample. This technique enabled mono-acyl histidine to be eluted at a position on the chromatogram which did not coincide with any other amino acid derivative. The amount of butanol necessary for the quantitative conversion of di-acyl histidine to the mono-acyl derivative must be determined for each instrument.

A dual column chromatographic system of EGA and OV-17 as the stationary phases was prepared from which all twenty of the protein amino acids were quantitatively eluted and separated in 55 min. The quantitative GLC analysis of the amino acids in ribonuclease is reported. This chromatographic system was very stable, and recent studies with these columns have shown that excellent results can be obtained for samples containing only 1 μg of each amino acid with 50 ng of each being injected. Further studies have shown that this system also permits a semiquantitative analysis of samples containing 100 ng of each amino acid, with 5 to 10 ng being injected.

It is concluded that amino acids can be quantitatively determined in proteins, peptides, and biological substances by gas–liquid chromatography. The subject of a paper in preparation covers the applications and details of macro, semimicro and micro methods.

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QUANTITATIVE DETERMINATION OF N-ACETYLNEURAMINIC ACID BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

An analytical method is described for preparing the trimethylsilyl derivative of N-acetylneuraminic acid (NANA) for quantitative gas–liquid chromatographic analysis. Satisfactory quantitation (RSD 1.6 %) on 200 μg amounts of NANA was obtained from a 2 % OV-17 column and flame ionization detector using an internal standard of *trans*-stilbene. The minimal detectable amount (MDA) was found to be about 8 ng of standard injected. The silylation of NANA was carried out in acetonitrile as solvent at 125° for 2 h using BSTFA as silylation reagent.

This procedure was successfully applied to the determination of NANA in K-casein (*ca.* 2 %). The NANA was hydrolyzed from the protein in 0.05 *N* HCl at 80° for 1.5 h and extracted with CH_3OH prior to silylation. These hydrolysis conditions were found to provide the maximum recovery of NANA from K-caseins. Any decomposition of NANA resulted in the appearance of an additional chromatographic peak in the 180° region of the chromatogram. The sialic acid values obtained by the proposed method were about 10 % higher than those of WARREN's colorimetric method. This is due in part to the hydrolysis conditions employed by the latter method. Two hundred microgram quantities of NANA were determined in K-casein by the proposed method with a relative standard deviation of 3.8 %.

An interesting by-product of this research was the appearance of a series of three compounds in the 200° region of the gas chromatograms of α_{s_1}- and β_s-caseins but not in K-casein. Identification of these compounds might benefit casein research with respect to the calcium sensitivity of the caseins (α_{s_1}- and β-) in which they are present.

This gas–liquid chromatographic method is offered as a more selective and probably more accurate alternative for determining sialic acids in biological samples and glycoproteins. It also demonstrates the capabilities of quantitative gas–liquid chromatographic analysis of higher molecular weight compounds.

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** Experimental data are taken in part from doctoral thesis, University of Missouri, June, 1968; NASA predoctoral research fellow.

A more selective method was desired for the quantitative determination of sialic acids. The sensitivity (10 to 60 μg) of WARREN's assay[8] for sialic acids is excellent and only a few naturally-occurring compounds interfere. The chromogen in this procedure is β-formyl-pyruvic acid, which results from the periodate oxidation of compounds in the class of 2-keto 3-deoxy-sugars. However, the 2-deoxy-sugars (*e.g.*, 2-deoxyribose) yield malonaldehyde on periodate oxidation, which gives a color with 2-thiobarbituric acid. The absorption spectrum (max. 532 mμ) for the malonaldehyde chromophore overlaps that of β-formyl-pyruvic acid (max. 549 mμ). This necessitates a correction for 2-deoxyribose in WARREN's sialic acid procedure. In addition, since only the first four carbon atoms in the neuraminic acid molecule are involved in the color development, the procedure does not differentiate the various neuraminic acid derivatives (N-acetyl-, N-glycolyl- and N,O-diacetyl-).

The introduction of the trimethylsilyl group for making volatile TMS derivatives has made possible the analysis of higher molecular weight compounds by gas–liquid chromatography[2,4,5]. The TMS derivative has found very successful application in determining, by GLC, a large number of carbohydrate compounds[6], and BENTLEY *et al.*[1] reported obtaining a gas chromatographic peak for N-acetylneuraminic acid (NANA).

This paper reports the results of an investigation on the development of a quantitative assay method for NANA by gas–liquid chromatography using the TMS derivative. After accomplishing this with standard materials the method was applied to determining the NANA content of K-casein (*ca.* 2% NANA) and comparing this to colorimetric values. In addition, a further investigation was made into the effects of mild acid hydrolysis on NANA. The optimum working range of the method is 200–800 μg of NANA, however good results can be obtained at the 100 μg level.

EXPERIMENTAL

Instrumentation and reagents

An F and M Model 401 gas chromatograph and flame ionization detector was used. The gas chromatograph was equipped with a L and N Speedomax G recorder and Disc integrator, Model 203. The electrometer was a Barber Coleman, Model 5042-1.

Chromatographic conditions

Column temperature initial, 175°; final, 260°
Program rate 7.9°/min
Attenuation 1 K
Carrier flow, N_2 54 ml/min
Air (to detector) 350 ml/min
Hydrogen (to detector) 38 ml/min
Chart speed $^1/_3$ in./min.

A U-shaped borosilicate glass column packed with a substrate of 2.0 w/w% OV-17 on High Performance Chromosorb G was used for the chromatography. The OV-17 was obtained from Applied Science Labs, Inc., State College, Pa., and Chromosorb G was from Varian Aerograph, Walnut Creek, Calif. Nanograde acetonitrile

from Mallinkrodt Chemicals, St. Louis, Mo., was the silylating solvent. The silylating reagent, BSTFA ("Regisil" concentrate) was purchased from Regis Chemical Company, Chicago, Ill. Crystalline synthetic N-acetylneuraminic acid obtained from Sigma Chemicals, St. Louis, Mo., was used as the standard.

Procedures

A standard solution was prepared by dissolving 20 mg of NANA and 20 mg of *trans*-stilbene (I.S.) in 100 ml of reagent-grade CH_3OH giving a final concentration of 200 μg/ml of each. The desired amounts of standard solution were transferred into screw-topped culture tubes (Pyrex No. 9826, 16 × 75 mm) and taken to dryness on a roto-evaporator at 60°. During evaporation the tubes should be placed in a nearly vertical position to prevent coating of the material over a large interior surface area of the glass. One milliliter of acetonitrile and 0.3 ml of BSTFA were then added to the samples. The samples were mixed thoroughly and placed in a 125° oil bath for 2 h. They were shaken periodically for the first 5–10 min of heating. A good working concentration range is from 200–800 μg NANA/ml acetonitrile. It was necessary to dissolve the standards in CH_3OH rather than CH_3CN because of the low solubility of NANA in acetonitrile. After silylation the samples were allowed to come to room temperature, then injected (6 to 7 μl) in sufficient quantity to give a response of 500 to 600 counts.

To release quantitatively the NANA from the protein, 10 mg samples of K-casein were hydrolyzed in 2 ml of 0.05 *N* HCl for 1.5 h at 80°. After hydrolysis the samples were taken to visible dryness on a lyophilizer (approximate time: 7 h). NANA was extracted with 2 ml of warm (60°) methanol containing an approximately equal weight (NANA/I.S., 1:1) of *trans*-stilbene as internal standard. The extraction was conducted in the following manner. After adding the 2 ml of CH_3OH the samples were mixed for 5 to 10 min on a Vortex mixer, then centrifuged on a clinical centrifuge at 2,000 r.p.m. for 10 min and decanted into identical tubes avoiding the transfer of the insoluble protein. The extracts were then dried on a roto-evaporator in the same manner as described for the standards, then silylated in 1 ml of acetonitrile and 0.5 ml of BSTFA for 2 h at 125°. Excess BSTFA was used to react with the soluble protein that was carried over in the extraction. The concentration of NANA in the extract was about 200 μg/ml. It was necessary to extract NANA in this way to eliminate interference that would be caused during silylation by the fifty-fold excess of protein present.

Calculations

$$\text{(1) Relative weight response for NANA/I.S.} = \frac{\text{Area NANA}}{\text{Area I.S.}} \times \frac{\text{wt. I.S.}}{\text{wt. NANA}}$$

$$\text{(2) Grams of NANA} = \frac{\text{Area NANA} \times \text{wt. I.S.}}{\text{Area I.S.} \times 1.118}$$

RESULTS AND DISCUSSION

The *trans*-stilbene (retention temperature 210°) and NANA (retention temperature 255°) peaks are shown on the chromatogram in Fig. 1. With respect to

solubility, acetonitrile is a poor solvent for NANA but it was the only solvent in which a single chromatographic peak was obtained for NANA; dimethylformamide, dimethylacetamide and other more polar solvents resulted in two peaks. The relative weight response (RWR) for NANA/*trans*-stilbene was 1.118 ± 0.018 (ten independent analyses) calculated from disc integration data. The standard curve for NANA at concentrations between 200 and 800 μg/ml shows good linearity (Fig. 2). The RWR varied slightly due to instrumental changes; thus, it was necessary to run standards and calculate the RWR for each series of samples.

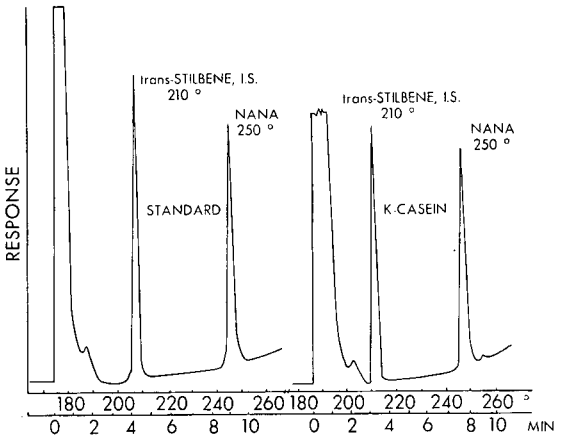

Fig. 1. GLC chromatograms of N-acetylneuraminic acid (NANA) and K-casein. Column: 2.0 w/w OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D. glass; initial temperature: 175°; program rate: 7.9°/min; N_2 flow rate: 54 ml/min. Each peak represents 1.4 μg.

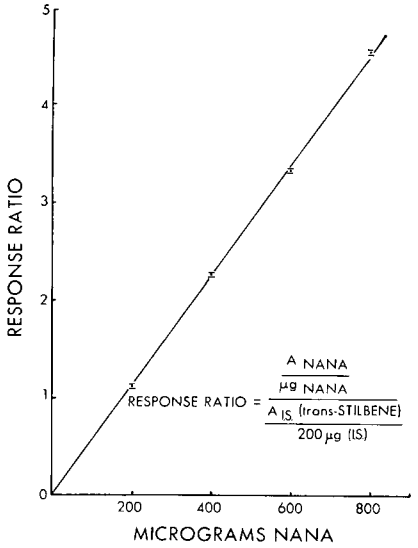

Fig. 2. N-Acetylneuraminic acid standard curve.

The effect of silylation temperature on RWR is shown in Fig. 3. Although considerably less silylation time is required at 150° it was not practical to use this high a temperature because of sample losses sustained from tube cap leakage. The silylated derivative showed good stability in closed tubes for periods up to one week. It was found that new rubber septa used for column injection produced peaks at chromatographic temperatures above 250° if they were not previously conditioned at high temperature for 24 h. Performance blanks containing only acetonitrile and BSTFA were analyzed periodically for extraneous chromatographic peaks and none were found.

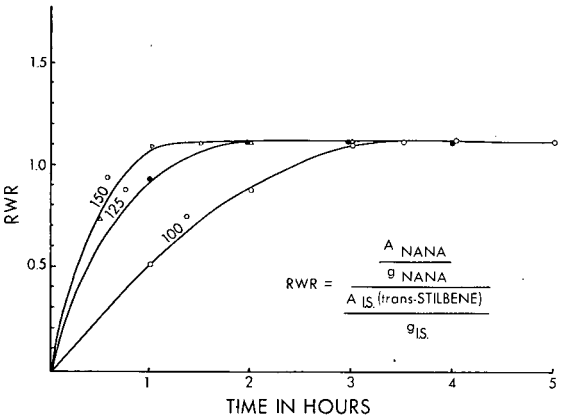

Fig. 3. Response as a function of silylation time and temperature.

The effects of acid hydrolysis on NANA were investigated and it was found that the RWR of the NANA standards decreased over 5 % after hydrolysis in 0.1 *N* HCl, 80°, for 1 h (Table I). The degradation of NANA was critically dependent on acid concentration. Decomposition of NANA was observed directly from the appearance of a new peak in the 180° region of the chromatograms. Lower concentrations of acid showed no effect on breakdown of NANA standards but low recoveries were obtained

TABLE I

RECOVERY OF N-ACETYLNEURAMINIC ACID AS A FUNCTION OF HYDROLYSIS TEMPERATURE, TIME, AND ACIDITY

	Temperature (°C)	*Time (h)*	*Acidity (N HCl)*	*% recovery*
NANA standard	60	1	0.2	86
	80	1	0.1	94
	80	1	0.75	95
	80	1.5	0.75	94
	80	1	0.05	101
	80	1.5	0.05	97
K-Casein[a]	80	1	0.05	87
	80	1.5	0.05	98
	80	2.0	0.05	96

[a] Based on NANA recovery from 0.05 *N* HCl, 80° and 1.5 h hydrolysis.

from the K-casein samples, indicating incomplete hydrolysis of NANA from the protein. From these experiments, the optimum hydrolysis conditions considering both parameters, loss due to decomposition of NANA and low values due to incomplete hydrolysis from the protein, were found to be 1.5 h, 80°, in 0.05 *N* HCl.

The GLC method was then applied for the analysis of sialic acid in an electrophoretically pure preparation of K-casein. The sample was found to contain 2.30 ± 0.09 w/w % NANA (Fig. 1, Table II). Table III gives the results obtained from the same K-casein by WARREN's method.

TABLE II

DETERMINATION OF SIALIC ACID IN K-CASEIN BY GAS–LIQUID CHROMATOGRAPHY

	Sample weight (mg)	*Area ratio NANA/I.S.*[a]	*Wt. NANA (μg)*	*% NANA*
NANA standard	0.200	1.000	200	100
	0.400	2.018	400	100
	0.600	2.964	600	100
K-Casein[b, c]	10	1.227	245	2.45
	10	1.194	239	2.39
	10	1.144	229	2.29
	10	1.096	219	2.19
	10	1.156	231	2.31
	10	1.100	220	2.20
	10	1.123	225	2.25
	10	1.193	239	2.39
	10	1.118	224	2.24
		Average 2.30 ± 0.09		

[a] Corrected so that equal weights of NANA and I.S. give equal areas (*i.e.*, area I.S. × 1.118).
[b] 0.200 mg I.S. added to samples.
[c] All hydrolyzed in 0.05 *N* HCl, 1.5 h, 80°.

TABLE III

DETERMINATION OF SIALIC ACID IN K-CASEIN BY WARREN'S METHOD

	Sample (μg)	*549 mμ O.D.*	*NANA (μg)*	*% NANA*
NANA standard	10.8	0.460	10.8	100
	16.2	0.715	16.2	100
	21.6	0.940	21.6	100
K-Casein[a]	10^3	0.860	19.7	1.97
	10^3	0.870	19.9	1.99
K-Casein[b]	10^3	0.900	20.7	2.07
	10^3	0.880	20.2	2.02
K-Casein[c]	10^3	0.910	20.9	2.09
	10^3	0.910	20.9	2.09

[a] Hydrolyzed in 0.1 *N* H_2SO_4, 1 h, 80°.
[b] Hydrolyzed in 0.1 *N* HCl, 1 h, 80°.
[c] Hydrolyzed in 0.05 *N* HCl, 1.5 h, 80°.

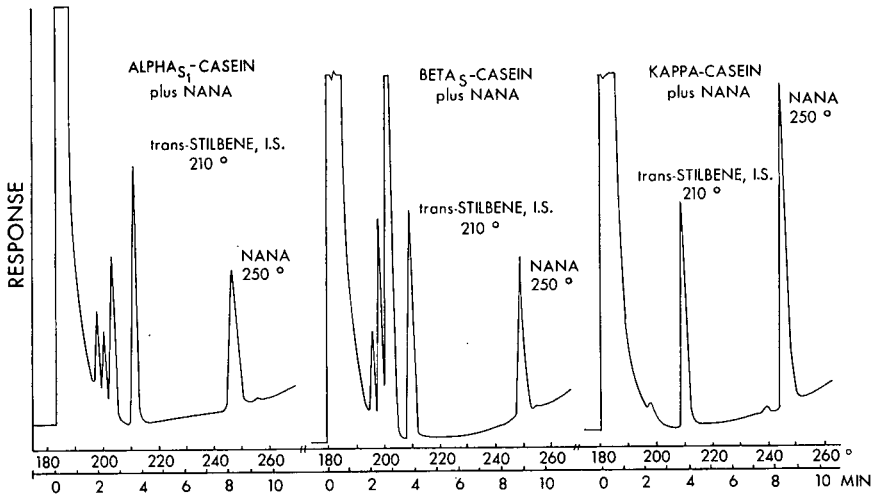

Fig. 4. GLC chromatograms of α_{s_1}-, β_s- and K-caseins with NANA added. Column: 2.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D. glass; initial temperature: 175°; program rate: 7.9°/min; N_2 flow rate: 54 ml/min. Each peak represents *ca.* 1.4 μg.

In recovery experiments, samples of α_{s_1}- and β-caseins, containing no NANA of their own, were spiked with NANA and carried through the hydrolysis, extraction, and derivatization steps. In all cases, a recovery of better than 97% was achieved (Fig. 4). K-Casein was also spiked with NANA and a single chromatographic peak was observed, thus confirming the presence of the N-acetyl derivative of neuraminic acid in K-casein. Although only the N-acetyl substituted sialic acid was investigated in this research, other sialic acids could be analyzed in mixtures by this method.

Three major peaks (retention temperature 190° to 200°) were observed in the chromatograms of the α_{s_1}- and β_s-caseins but not in K-casein. β_s-Casein showed a large concentration of the component having the highest retention temperature. Although histidine, arginine, and cystine have retention temperatures in this region of the chromatogram, these peaks probably do not represent amino acids, otherwise, they would appear in K-casein as well. These are more than likely other types of carbohydrates known to be present in caseins. It is interesting to note that these compounds are present in the calcium-sensitive caseins, α_{s_1}- and β_s-, but not K-casein.

The minimal detectable amount (MDA), defined as the amount of substance needed to produce a signal to noise ratio of 2:1, was determined for the silylated NANA derivative and was found to be about 8 ng.

The OV-17 chromatographic column was found to be quite stable for several weeks of continuous use although the operating temperatures exceeded 260°. BSTFA was used as silylation reagent because its solubility in acetonitrile far exceeds that of ordinary BSA.

The colorimetric method of analysis gave lower values for NANA in K-casein than those obtained by gas–liquid chromatography. Obviously some of the NANA is decomposed by 0.1 *N* H_2SO_4 hydrolysis, even though NANA was reported to be stable under these conditions[7]. The colorimetric method probably is less sensitive to the partial decomposition of NANA because only a fragment of the molecule is re-

quired for color development; whereas, in the proposed method the entire molecule is derivatized. The GLC method has the advantage of selectivity, and is a convenient method for determining unbound and protein-bound sialic acids.

This procedure offers certain definite advantages over the colorimetric methods. Lower molecular weight carbohydrates do not interfere and the various neuraminic acid derivatives would be distinguishable from one another. This research also demonstrates the capability of gas–liquid chromatography as an analytical tool for higher molecular weight compounds. By controlling the extent of hydrolysis, a complete and rapid analysis of the amino acid and non-protein components; *e.g.*, carbohydrates, nucleic, acid constituents[3] and phosphate can be achieved on a single sample.

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CHROM. 3696

BESTIMMUNG DER SORPTIONSKAPAZITÄT VON POLYAMIDPULVER ZUR SÄULENCHROMATOGRAPHIE

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SUMMARY

Determination of the adsorption capacity of polyamide powder in column chromatography

A procedure has been described for determining the adsorption capacity of polyamide powder. Starting from a known concentration of a solution of β-naphthol orange, the adsorption capacity of polyamide powder can be measured by determination of the adsorbed and non-adsorbed dyes by means of a colorimetric method. The results show that commercial powders have various adsorption capacities.

In den letzten Jahren werden neben den herkömmlichen anorganischen Sorptionsmitteln wie Kieselgel und Aluminiumoxid, immer häufiger rein organische Träger zur Chromatographie verwendet, darunter die Polyamide vom Typ des Perlons und Nylons. Seit den ersten Versuchen zur Bestimmung von Gerbstoffen an Polyamid durch BATZER[1] und den Arbeiten von CARELLI *et al.*[2] und GRASSMANN *et al.*[3,4] vor über zehn Jahren, hat sich die Polyamidchromatographie inzwischen besonders auf dem Dünnschichtgebiet sprunghaft entwickelt.

Die Wirkung der Polyamidchromatographie, ein ungewöhnlicher Typ der Verteilungschromatographie[2], ist durch die Möglichkeit von Protonbrückenbindungen[5] bedingt, wobei die Fliessmittel mit den zu trennenden Substanzen um die Ausbildung von Protonbrücken[6] konkurrieren. Durch den Ausdruck Chemisorptionschromatographie wird u.E. diese gut gekennzeichnet.

Polyamide als Polykondensate mit linearem Kettenaufbau enthalten als strukturbestimmende Elemente Carbonamidgruppen, die in fortlaufender Gruppierung durch mehr oder weniger lange CH_2-Ketten miteinander verbunden sind. Diese Ketten sind orientiert und mit benachbarten Ketten durch Protonbrückenbindungen zu Lamellen verknüpft, wobei N–H- und O=C-Gruppen zu Protonbrückenbindungen eingerastet sind. In diesem Zustand kann das Polyamid aber auch als Pulver nur ein geringes Adsorptionsverhalten besitzen, wie aus Arbeiten von BONDARCZUK[7] bestätigt wird. Er zeigte durch Anfärben von Polyamidfasern mit unterschiedlichen Verstreckungsgraden, dass Fasern mit einer hohen Orientierung der Makromoleküle

weniger anfärbbar sind als solche, die nur gering verstreckt wurden. Die Fig. 1 zeigt den Verlauf der Sorptionsisothermen in Abhängigkeit vom Orientierungsgrad.

Zur Herstellung eines geeigneten Sorptionsmittels werden die in den Makromolekülen bestehenden Protonbrücken durch stark polare Lösungsmittel aufgehoben, gelöst und mit Wasser das Polyamid wieder ausgefüllt. Dieses Material mit vielen unorientierten Ketten und dadurch unbeanspruchten N–H- und O=C-Gruppen, das einen hohen Anteil amorpher Bereiche aufweist und deshalb mit Wasser quillt, besitzt die besten chromatographischen Eigenschaften. Diese hängen von der Korngrösse und den unterschiedlichen amorphen Bezirken ab.

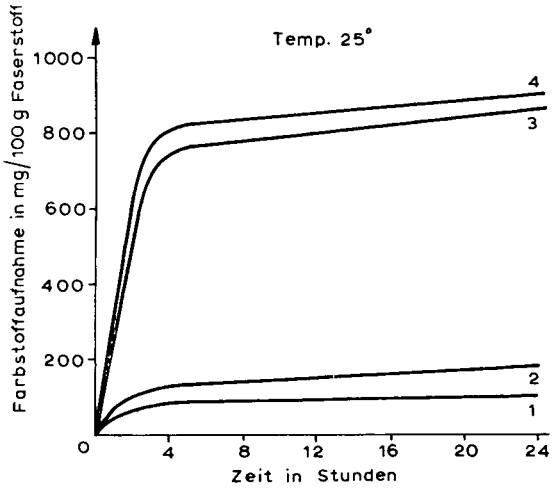


Fig. 1. Farbstoffaufnahme von Polyamid in Abhängigkeit vom Verstreckungs- bzw. Orientierungsgrad. (1) Verstreckungsgrad 400%; (2) Verstreckungsgrad 300%; (3) Verstreckungsgrad 120%; (4) Verstreckungsgrad 0%.

Der chemische Aufbau des Polykondensates kann auch einen Einfluss auf das Verhalten ausüben. Im Nylon 66 können die meisten Carbonamidgruppen, bedingt durch den linearen Bau, mit benachbarten Ketten Brückenbindungen eingehen, während Nylon 56, ein Polypentamethylenadipinatmischkondensat, dagegen nur ungefähr die Hälfte der Gruppen zur Protonbrückenbindung zur Verfügung stellen kann. Durch den strukturellen Aufbau stehen daher auch im verstreckten Material mehr aktive Zentren zur Verfügung.

Mit sauren und besonders phenolischen Verbindungen gehen die Sauerstoffatome des aufgeweiteten unorientierten Polyamids besonders leicht Protonbrückenbindungen ein. Diese Eigenschaft hat das Polyamid mit den natürlichen Proteinfasern Wolle und Seide gemeinsam; bei gewissen Wollfarbstoffen, in der Lederhautgerbung und zur Anreicherung von Lebensmittelfarbstoffen nach der Wollfadenmethode wird davon Gebrauch gemacht.

Gegenüber anorganischen Sorptionsmitteln besitzt Polyamidpulver einige beachtliche Vorteile:

(1) die hohe Sorptionskapazität gestattet eine vorteilhafte präparative Säulenchromatographie,

(2) auf Grund der hohen Kapazität kann es zur Abtrennung unerwünschter Begleitstoffe in der Analytik dienen und

(3) erlaubt die günstige Kapazität auch die Anwendung geringer Mengen Sorptionsmittel in Mikrosäulen für zeitsparende quantitative Bestimmungen[8].

Wenn man die bisher erschienenen Arbeiten auf dem Gebiete der Polyamidchromatographie kritisch betrachtet, so lassen sich folgende Tendenzen erkennen:

(1) Die anfangs anteilmässig überwiegenden Arbeiten auf dem Gebiete der Säulenchromatographie sind zu Gunsten der Dünnschichtchromatographie wesentlich zurückgegangen.

(2) Während früher vorwiegend Substanzen mit phenolischen Hydroxylgruppen in polaren Fliessmitteln getrennt wurden, werden jetzt auch andere Verbindungsklassen mit unterschiedlichen Fliessmittelkombinationen verwendet. Das trifft besonders für die Dünnschicht-Chromatographie zu.

(3) Es zeichnen sich Verfahren ab, die sich des Polyamids nicht zur reinen Chromatographie, sondern als Adsorbens zur Entfernung störender Begleitstoffe mit sauren funktionellen Gruppen bedienen.

Wir beobachteten, dass unter den im Handel befindlichen Polyamidpulvern verschiedener Herkunft grosse Unterschiede im Adsorptionsverhalten gegenüber Farbstoffen bestehen. Saure Azofarbstoffe können stellvertretend für die zur Adsorption an Polyamide in Frage kommenden Pflanzeninhaltsstoffe stehen, wie wir uns an Hand der Chlorogensäure überzeugen konnten.

In der Literatur ist unseres Wissens bisher über ein unterschiedliches Verhalten von Polyamidpulver zur Chromatographie bis auf die lapidare Bemerkung von WEINGES *et al.*[9] "mit käuflichem Perlonpulver wurde keine Trennung erzielt", weder berichtet worden, noch liegen Ergebnisse vergleichender Untersuchungen der Adsorptionseigenschaften von Polyamidpulver unterschiedlicher Herkunft, Herstellung oder Aufbaus vor. Dagegen führen HÖRHAMMER UND WAGNER[10] an, dass für die Chromatographie von Flavonen und Isoflavonen grundsätzlich alle Perlon- oder Nylonpulver, sofern sie frei von monomeren Anteilen sind und eine mittelere Korngrösse von *ca.* 100 μ besitzen, geeignet sind. Für quantitative analytische Bestimmungen empfehlen sie allerdings ein "standardisiertes" Präparat, ohne jedoch anzugeben, wie eine Standardisierung durchzuführen wäre oder welche charakteristischen Merkmale ein solches Polyamid aufweist.

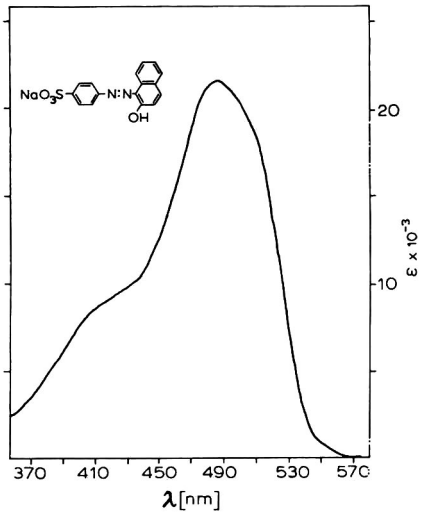
Wenn Polyamidpulver zur Chromatographie vorgeschlagen wird oder zur Behandlung von Getränken Mengenangaben gemacht werden[11–15], um eine bestimmte Wirkung zu erzielen, muss dabei beachtet werden, dass eine höhere Zugabe von Sorptionsmittel die Getränke unansehnlich, farbarm und geschmacklich fade werden lassen kann. Daher muss die Adsorptionsfähigkeit des empfohlenen Materials bekannt sein. Nur dann können reproduzierbare Ergebnisse erzielt werden, wenn das Polyamidpulver über eine bestimmte Kapazität verfügt.

In Anbetracht der Wichtigkeit für die gesamte Polyamidchromatographie an Säulen hielten wir es für erforderlich, eine schnelle und einfache Möglichkeit zur Bestimmung der Sorptionseigenschaft des Polyamidpulvers zu entwickeln, die (a) einen Aussagewert für die vorgesehene Verwendung besitzt, (b) für das Sorptionsmittel charakteristisch ist und (c) sich leicht bestimmen lässt.

Wir wählten hierfür die Sorptionskapazität, die aussagt, welche Menge Substanz ein Adsorbens zu adsorbieren vermag. Als Mass soll die Anzahl der adsorbierten

Mikromole Testsubstanz dienen, die von einem Gramm Sorptionsmittel festgehalten werden. Saure Azofarbstoffe sind für eine solche Kapazitätsbestimmung geeignet, da sie vom Polyamid gut adsorbiert werden. ENDRES, GRASSMANN UND OPPELT[5] verwendeten dieses Adsorbens schon zur Affinitätsbestimmung von Azofarbstoffen, wobei eine Abhängigkeit der Wanderungsgeschwindigkeit von der Zahl der Sulfogruppen im Molekül gefunden wurde. Sowohl in dieser Veröffentlichung, als auch in einer Arbeit über die Aufstellung von Sorptionsisothermen nitrierter aromatischer Verbindungen zur Untersuchung des Haftmechanismus an Polyamid[16] wird über ein unterschiedliches Verhalten von Polyamid verschiedener Herkunft nicht berichtet.

Für eine Kapazitätsbestimmung wählten wir den Azofarbstoff β-Naphtholorange (Orange II) aus, der leicht herstellbar und gut wasserlöslich ist, sowie über eine gute Affinität zum Polyamid verfügt. Ausserdem lässt sich eine wässrige Lösung des Farbstoffs durch Messung der Lichtadsorption schnell und mit hinreichender Genauigkeit photometrisch auswerten. Die Fig. 2 zeigt den Verlauf des Absorptionsspektrums des Farbstoffs mit seinem Absorptionsmaximum bei 485 nm.



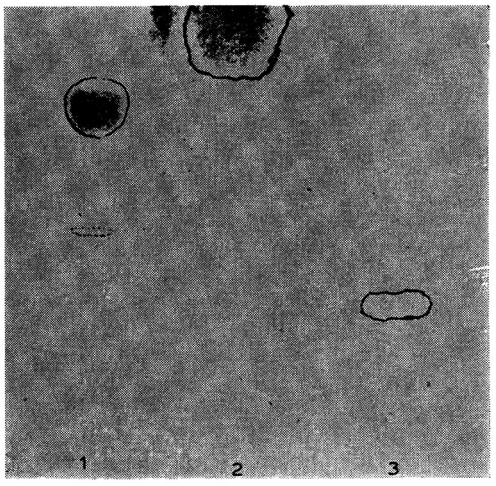


Fig. 2. Absorptionsspektrum vom β-Naphtholorange in Wasser; $\lambda_{max} = 485$ nm.

Fig. 3. Cellulose-Dünnschichtchromatogramm vom β-Naphtholorange (1), β-Naphthol (2) und der Sulfanilsäure (3). Fliessmittelsystem: *n*-Butanol–Eisessig–Wasser (4:1:5, organische Phase).

Aus dem Dünnschichtchromatogramm der Fig. 3 geht hervor, dass der selbst dargestellte Farbstoff nach dem Umfällen aus methylalkoholischer Lösung mit Chloroform weitgehend frei von Nebenprodukten ist.

Zur Bestimmung der Sorptionskapazität wurde das Polyamidpulver mit Wasser angequollen, abfiltriert und auf dem Filter so lange mit Wasser ausgewaschen, bis alle monomeren und niedermolekularen Anteile entfernt waren. Danach wird das getrocknete Adsorbens mit der Farbstofflösung bekannter Konzentration behandelt, in ein Mikrochromatographierohr übergeführt und vom nichtadsorbierten Farbstoff durch Auswaschen mit Wasser befreit. Die vom Polyamid adsorbierte und alkalisch eluierte Farbstoffmenge wurde ebenso wie der nichtadsorbierte Farbstoff photo-

metrisch bestimmt, wobei die Summe beider Messungen die Ausgangskonzentration ergeben soll. Es konnte in allen Fällen der Farbstoff quantitativ wiedergefunden werden. Das spricht dafür, dass die Verluste während der Chromatographie zu vernachlässigen sind und dass die Methode gut reproduzierbar ist.

MATERIAL UND METHODEN

Reagenzien

β-Naphthol, Sulfanilsäure, $NaNO_2$, HCl, NaOH, Chloroform, Methanol, *n*-Butanol, Eisessig, Cellulosepulver (MN 300 UV), Seesand, Watte, Äthylalkohol 90 %ig.

Spezielle Geräte

Dünnschichtchromatographische Ausrüstung von DESAGA (Heidelberg); Herstellung der Cellulosedünnschichten wie üblich[17] oder im Handverfahren durch Schütteln von 13 g Cellulosepulver mit 65 ml 90 %igem Äthylalkohol (vergällt mit 1 % Petroläther) in einem verschlossenen Erlenmeyerkolben und Ausgiessen auf die Platte[18]. Mikrochromatographierohre (Normschliff Glasgeräte, 6980 Wertheim, Deutschland).

Untersuchungssubstanzen

Polyamidpulver der Firmen Macherey, Nagel und Co.; Woelm; Roth; Bayer; BASF.

Herstellung des Farbstoffes

Die Herstellung des β-Naphtholorange erfolgt in üblicher Weise[19], die Reinigung durch Auflösen in Methylalkohol und Ausfällen mit Chloroform. Vierhundert Milligramm des getrockneten Farbstoffs werden in 500 ml dest. Wasser gelöst. Drei Milliliter dieser Lösung, die 2.4 mg β-Naphtholorange entsprechen, in 50 ml Wasser besitzen eine Extinktion von 2.3 bei 485 nm (Ergebnis aus fünf Einzelmessungen).

Vorbereitung der Polyamidproben

Man lässt die Pulverproben 2 Std. lang mit dest. Wasser quellen, filtriert das Pulver ab und wäscht es mit *ca.* 200–300 ml dest. Wasser aus, um monomere und niedermolekulare Anteile sowie das Feinstkorn zu entfernen. Wenn diese Beimengungen nicht restlos entfernt werden, kann die spätere photometrische Bestimmung durch die kleinen Partikel sehr gestört werden.

Das ausgewaschene Pulver wird anschliessend im Trockenschrank bei 70° 24 Std. lang getrocknet; nach dem Abkühlen werden von jedem Muster jeweils drei Proben zu 100 mg abgewogen.

Durchführung

Jede Probe von 100 mg wird mit 3 ml der Farbstofflösung versetzt und 2 Std. lang unter öfterem Umschütteln auf den Farbstoff einwirken gelassen. Wie aus Vorversuchen ermittelt wurde, ist nach dieser Zeit die Sättigung des Polyamids erreicht. Nun überführt man das Gemisch in vorbereitete Mikrochromatographierohre (Fig. 4), lässt die Farbstofflösung in einen 50 ml fassenden Messkolben laufen und wäscht die

Säulenfüllung so lange mit dest. Wasser (10–20 ml) nach, bis dieses farblos abläuft. Nach dem Auffüllen zur Marke wird die vom Polyamidpulver nicht adsorbierte Farbstoffmenge photometrisch gegen eine entsprechend bereitete Kompensationslösung bestimmt.

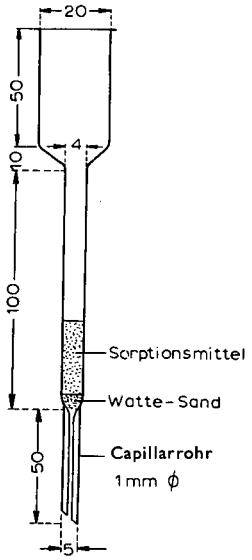


Fig. 4. Mikrochromatographierohr.

Zur Ermittlung der adsorbierten Farbstoffmenge wird die Säule mit *ca.* 10 ml methylalkoholischer Natriumhydroxidlösung (1 g NaOH in 1 l 70 %igem Methylalkohol) zur Desorption des Farbstoffes eluiert und das Eluat in einem 50 ml fassenden Messkolben, in den 0.5 ml verd. Essigsäure (1 Teil Eisessig + 1 Teil 70 %igem Methylalkohol) vorgelegt wurde, aufgefangen. Nach Auffüllen des Volumens zur Marke wird die eluierte Farbstoffmenge ebenfalls durch Bestimmung der Extinktion bei 485 nm gegen eine entsprechend bereitete Kompensationslösung gemessen. In einigen Fällen ist es erforderlich, die resultierenden Farbstofflösungen evtl. zu verdünnen, um in den einwandfrei messbaren Extinktionsbereich zu kommen.

Aus den erhaltenen Extinktionswerten der adsorbierten und nichtadsorbierten Farbstoffmenge wird die Sorptionskapazität ermittelt, indem die Mikromole des adsorbierten Farbstoffs errechnet werden, die von 1 g Polyamidpulver festgehalten wurden (β-Naphtholorange MG 350,3).

Durch Bestimmung der Summe des adsorbierten und nichtadsorbierten Farbstoffs und Vergleich mit der eingesetzten Farbstoffmenge wird die einwandfreie Versuchsführung überwacht.

ERGEBNISSE UND DISKUSSION

Durch die Adsorption von β-Naphtholorange an Polyamidpulver lässt sich die Sorptionskapazität dieses Adsorbens sehr gut bestimmen. Als ein Sorptionsmittel, das für die Polyamidchromatographie gut geeignet ist, sehen wir ein Material an, das über

eine Sorptionskapazität von 30–70 μMole Farbstoff/g Polyamidpulver verfügt. Höhere Kapazitäten sind erwünscht, niedere schränken die Anwendungsmöglichkeit sehr stark ein.

In der Tabelle I sind die aufgefundenen Sorptionskapazitäten von achtzehn verschiedenen Mustern Polyamidpulver zusammengestellt.

Die gemessene Kapazitäten stimmen mit den bei ENDRES UND HÖRMANN[20] gemachten Angaben nur in zwei Fällen der Testreihe überein, die meisten Proben besitzen eine wesentlich geringere Sorptionskapazität.

TABELLE I

SORPTIONSKAPAZITÄTEN VON POLYAMIDPULVER

PA = Polyamidpulver.

Material	*Farbstoff*				
	Zugesetzt	*Nichtadsorbiert*	*Adsorbiert*		
	(E)	*(E)*	*E*	*mg/100 mg PA*	*μMole/g PA*
BASF Divergan SZ	2.300	2.207	0.135	0.139	3.9
BAYER Durethan BK 31	2.300	2.125	0.150	0.155	4.4
MN SC < 0.160 mm	4.600	0.485	4.120	4.256	121.2
MN SC < 0.070 mm	2.300	1.307	0.998	1.030	29.4
MN SC 6 (2117)	2.300	1.090	1.235	1.276	36.4
MN SC 66	2.300	0.407	1.930	1.995	57.0
MN SC 6 < 0.160 mm	2.300	1.480	0.863	0.892	25.5
MN SC 6 < 0.070 mm	2.300	0.260	1.980	2.040	58.3
MN SC 6 < 0.070 mm	3.200	0.821	2.398	2.461	70.4
ROTH 2-9109	2.300	2.200	0.166	0.172	4.9
ROTH	2.300	2.173	0.153	0.158	4.5
WOELM SC (1965)	2.300	0.385	1.930	1.991	56.9
WOELM SC 177	2.300	0.898	1.426	1.472	42.1
WOELM SC (1966)	2.300	0.349	1.904	1.970	56.3
WOELM SC (1968)	2.300	0.958	1.348	1.391	39.8
WOELM SC 158	2.300	1.375	0.980	1.012	28.9
WOELM SC 327	2.300	0.862	1.475	1.521	43.6

Aus den Angaben der Tabelle I ist ersichtlich, dass sehr grosse Unterschiede in der Kapazität von Polyamidpulver bestehen können und dass auch Material vom gleichen Hersteller, das unter der gleichen Bezeichnung im Handel ist, chargenweis ein unterschiedliches Adsorptionsverhalten besitzt. Offenbar ist die Herstellung einer stets gleichbleibenden Qualität beim Polyamid schwierig.

Die Ergebnisse zeigen aber auch, dass einwandfreie Polyamidpulver über eine beachtliche Sorptionskapazität verfügen, die mit *ca.* 100 μMole/G Pulver weit über diejenige der üblichen Adsorbentien liegt, von denen ohne Überladung nicht mehr als nur einige Mikromole von der gleichen Menge Sorptionsmittel festgehalten werden können[20].

Wenn man ein Polyamidpulver geringerer Qualität in konz. Salzsäure löst und das Polyamid mit wässrigem Methylalkohol wieder ausfällt, kann daraus ein Pulver hergestellt werden, das über eine bessere Kapazität verfügt[17]. Das gegenüber Farbstoff am wenigsten wirksame Material der untersuchten Proben, das Divergan SZ 9010 wurde umgefällt. Die Tabelle II zeigt die erzielte Verbesserung der Kapazität.

Wie aus den Angaben hervorgeht, kann aus einem Polyamidpulver niedriger Kapazität durch Umfällen eine Qualität mittlerer Sorptionskapazität auf eine einfache Weise hergestellt werden.

Zur Überprüfung haben wir die Sorptionskapazität auch mit einer anderen Substanz bestimmt, wobei wir auf die gleiche Menge Polyamidpulver (100 mg) eine Chlorogensäurelösung bekannter Konzentration einwirken liessen und die adsorbierte und nichtadsorbierte Menge dieses Naturstoffes durch Messung der Lichtabsorption bei 324 nm bestimmten. Am Beispiel des Polyamidmusters MN SC (Nr. 3) konnten wir zeigen, dass dieses gegenüber der Chlorogensäure eine Kapazität von 107 μMole/g besitzt, was grössenordnungsmässig mit dem Ergebnis des Farbstofftestes übereinstimmt und dass die Kapazität von der Art der zu adsorbierenden Verbindung unabhängig ist, sofern die für die Adsorption verantwortlichen funktionellen Gruppen in gleicher Zahl vorhanden sind. In dem Falle der Chlorogensäure sind die in *ortho*-Stellung zueinander stehenden phenolischen Hydroxylgruppen als nur eine chromatographisch wirksame Gruppe zu rechnen[21].

TABELLE II

VERBESSERTE SORPTIONSKAPAZITÄT VON POLYAMIDPULVER

Material	*Farbstoff*				
	Zugesetzt	*Nicht adsorbiert*	*Adsorbiert*		
	(*E*)	(*E*)	*E*	*mg/100 mg PA*	*μMole/g PA*
Divergan	2.300	2.207	0.135	0.139	3.9
Umgefällt	2.300	1.354	0.930	0.952	27.2

Die sich immer mehr ausweitende Anwendung des Polyamidpulvers in der chemischen Praxis und die sehr unterschiedlichen Qualitäten der Pulver des Handels und der Industrie rechtfertigen die Bemühungen um eine einfache und schnelle Bestimmung der für die Chromatographie und Analytik so überaus wichtigen Sorptionskapazität. Es scheint, dass die Beobachtungen über ein unterschiedliches Verhalten von Sorptionsmitteln nur für das Polyamid zutreffen, denn über anorganische Adsorbentien und Cellulosepulver zur Säulenchromatographie sind derartige Angaben nicht gemacht worden.

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ZUSAMMENFASSUNG

Durch Einwirkung von Farbstofflösungen bekannter Konzentration lassen sich die Sorptionskapazitäten von Polyamidpulver schnell und hinreichend genau bestimmen. Achtzehn verschiedene Muster Polyamid wurden untersucht, wobei unterschiedliche Kapazitäten gefunden wurden.

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CHROM. 3673

EXTRAKTIONSCHROMATOGRAPHISCHE TRENNUNG DER FREIEN UROPORPHYRINISOMERE I UND III UND DEREN SIMULTANE QUANTITATIVE BESTIMMUNG

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SUMMARY

Separation and simultaneous quantitative determination of the free uroporphyrin isomers I and III by means of extraction chromatography

Separation and quantitative determination of the uroporphyrin isomers I and III in the acid form can be performed simultaneously in the partition system tri-*n*-butylphosphate/1 *N* hydrochloric acid using columns with a large number of theoretical plates ($N = 300$–450). The eluent is passed through a flow cuvette and the transmission is recorded continuously. The transmission peaks of the isomers are digitized and transformed into extinction values. By integrating the extinction peaks, the amount of the uroporphyrins can be evaluated from the corresponding peak areas.

Using the mathematical procedure described the time necessary for separation ($t_{El.}$) can be shortened appreciably. This method allows the decomposition of complex elution peaks even at low distances of the peak centres ($\Delta x_p/\overline{T}_{50} = 1.5$–$2.0$, depending on the mass ratio) and unfavourable mass ratio ($0.06 \leqq$ UP-I/UP-III $\leqq 16$).

Under suitable conditions (length of the column: $L_s = 120$ cm; temperature of the column: $T_s = 15$–$30°$; concentration of the eluent: $c_{HCl} = 1\ N$) complete analysis of both isomers can be achieved in about 20–30 h. The quantitative determination can be performed up to approx. 1 μg with an accuracy of 10 % (95 % deviation). No appreciable losses of the uroporphyrins occur ($R = 99 \pm 5$ %) even under extreme conditions ($T_s = 60°$; $c_{HCl} = 3\ N$).

EINLEITUNG

Zur Diagnostizierung von Hämsynthesestörungen hat die Isomerenanalyse der Porphyrine eine besondere Bedeutung erlangt. Sie vermag darüber Aufschluss zu geben, in welchem Umfange die Bildung von Hämpräkursoren des Isomerentyps I, der zur Hämbildung nicht befähigt ist, erfolgt, und gestattet somit, die vorwiegend auf enzymatische Defekte zurückgehende Störung innerhalb dieser Reaktionskette festzulegen.

Während die Auftrennung der Coproporphyrinisomere I und III in freier[1-3] oder veresterter[4] Form keine methodischen Schwierigkeiten bereitet, erscheint die Trennung der Isomere I und III von Uroporphyrin, aufgrund deren ausgeprägten Neigung zur Aggregatbildung[5-7], nicht unproblematisch. Bei den in der Literatur bisher beschriebenen papierchromatographischen[5,8], säulenchromatographischen[6] und dünnschichtchromatographischen[9] Methoden ist stets Voraussetzung, dass beide Isomere in Esterform vorliegen. Diese Untersuchungen, die in einem Falle mit radioaktiv markierten Substanzen kontrolliert wurden[7], zeigten, dass eine vollständige Auftrennung der Uroporphyrinisomere nur schwer zu erreichen ist. Die stets auftretende Überlagerung der beiden isomeren Ester bei der Trennung, deren Ausmass sowohl von der applizierten Menge als auch von dem jeweiligen Isomerenverhältnis abhängt, wurde in einer sehr eingehenden Untersuchung bei der Dioxanmethode empirisch erfasst[5]. Die hierbei ermittelte mathematische Korrekturfunktion gestattet, diese Verluste innerhalb bestimmter Grenzen zu korrigieren. Die Methode unterliegt jedoch der Einschränkung, dass lediglich Uroporphyrinmengen um 1 μg bei einem Isomerenverhältnis von UP-I/UP-III $\leqq 5$ bei einer vertretbaren Fehlerspanne analysiert werden können.

Diese methodisch bedingten Schwierigkeiten lassen sich umgehen, wenn beide Uroporphyrinisomere in die entsprechenden Coproporphyrinisomere überführt werden[10], die als freie Säuren[1-3] oder auch als Ester[4] aufgetrennt und anschliessend quantitativ bestimmt werden können. Zur Bestimmung des Isomerenverhältnisses bei Uroporphyrin kann ebenfalls der Schmelzpunkt der Ester herangezogen werden[11]. Hierzu werden jedoch vergleichsweise grosse Porphyrinmengen benötigt, die überdies sehr sorgfältig präpariert werden müssen.

Über eine Trennung der freien Uroporphyrinisomere I und III wurde in der Literatur bisher nicht berichtet. In der vorliegenden Arbeit wird, anknüpfend an vorausgehende, grundlegende Voruntersuchungen[12,13], gezeigt, dass unter geeigneten Bedingungen die Isomere dieses Hämpräkursors in freier Form nicht nur aufgetrennt sondern darüber hinaus in einem Arbeitsgang auch gleichzeitig quantitativ bestimmt werden können. Zur Trennung werden mit Hostaflon C2/Tri-*n*-butylphosphat als Füllmaterial beschickte Säulen mit einer relativ hohen Anzahl theoretischer Trennböden ($N = 300$–450) eingesetzt, die sich bereits zur Uroporphyrin–Coproporphyrintrennung ausgezeichnet bewährten[13]. Die verwendete, weitgehend automatisierte Versuchsanordnung bedarf bei Durchführung der Auftrennung, die je nach dem vorliegenden Isomerenverhältnis 15–30 h in Anspruch nimmt, keinerlei Wartung.

Die Trennung der freien Säuren hat gegenüber der Ester den Vorzug, dass die über eine Filterbodenextraktion[12] aus dem Urin isolierten Porphyrine unmittelbar aufgetrennt und Verluste, wie sie in merklichem Umfange bei der Estertrennung aufgrund der partiellen Hydrolyse stets zu beobachten sind[5,6], hierbei praktisch vernachlässigt werden können[13].

EXPERIMENTELLER TEIL

Material

Uroporphyrin-I (UP-I). Das in Form des Oktamethylesters durch den Handel bezogene Uroporphyrin-I (Lieferfirma: Koch-Light Laboratories Ltd., Colnbrook. England) wurde zunächst in 5 *N* HCl verseift, dünnschichtchromatographisch ge-

reinigt und nach Elution mit 1 *N* HCl mit Tri-*n*-butylphosphat (TBP) extrahiert. Die Rückextraktion wurde mit 5 *N* HCl vorgenommen. Geringfügige TBP-Reste in der wässerigen Phase wurden durch mehrfaches Auswaschen mit 5 × 5 ml Benzol entfernt. Verluste an UP-I sind hierbei nicht zu befürchten. Die salzsäure Lösung des Hämpräkursors wurde auf eine Konzentration von 100 μg/ml 5 *N* HCl eingestellt und in dieser Form zu den einzelnen Untersuchungen eingesetzt. Sämtliche Pipettierungen erfolgten mit einer Aglaspritze, so dass die eingesetzten Porphyrinmengen exakt dosiert werden konnten. Die bei 4° im Kühlschrank aufbewahrte Stammlösung zeigte selbst über einen Zeitraum von einigen Wochen keinerlei Zersetzung.

Uroporphyrin-III (UP-III). Der ebenfalls in Esterform durch die Koch-Light Laboratories Ltd., Colnbrook, England bezogene Hämpräkursor ("*ex turaco* feathers") wurde, wie vorangehend für UP-I beschrieben, gereinigt und in salzsaurer Lösung bei einer Konzentration von 100 μg/ml 5 *N* HCl zu den Untersuchungen eingesetzt.

Coproporphyrin-I (CP-I). Coproporphyrin-I wurde aus UP-I durch Decarboxylierung nach der Methode von EDMONDSON UND SCHWARTZ[10] erhalten. Die Aufarbeitung wurde, wie nachstehend unter Methoden beschrieben, vorgenommen. Die CP-I-Konzentration der Stammlösungen schwankte, je nach Präparation, zwischen 20–50 μg/ml 5 *N* HCl.

Coproporphyrin-III (CP-III). Zur Darstellung von CP-III durch Decarboxylierung wurde von UP-III ausgegangen. Bei der Aufbereitung und Herstellung der Stammlösungen wurde, wie vorangehend für CP-I beschrieben, vorgegangen.

Benzol, p.A. Handelsübliches Produkt (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 1783).

Tri-n-butylphosphat (TBP). Als Extraktionsmittel wurde das im Handel erhältliche Produkt (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 8354) ohne weitere Reinigung eingesetzt.

Polytrifluorchloräthylen (Hostaflon C2). Das als Trägermaterial verwendete Hostaflon C2 wurde durch Aufbereitung des durch den Handel bezogenen Produktes (Hersteller: Farbwerke Höchst AG., Frankfurt-Höchst) mit einer Korngrösse von 50–100 μ erhalten. Die Imprägnierung mit dem Extraktionsmittel erfolgte durch tropfenweise Zugabe von TBP unter intensivem Rühren im Gewichtsverhältnis Hostaflon C2 : TBP = 2 : 1.

Salzsäure. Die zur Elution eingesetzten Salzsäurelösungen wurden durch Verdünnen aus konz. Salzsäure p.A. (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 317) erhalten. Das Elutionsmittel war stets mit TBP gesättigt und wurde vor jedem Versuch durch Auskochen (30 min) nochmals weitgehend entgast.

Methoden

Decarboxylierung von UP-I bzw. UP-III. Zur Darstellung der Coproporphyrinisomere I und III wurden UP-I und UP-III nach der Methode von EDMONDSON UND SCHWARTZ[10] decarboxyliert: 3 ml der salzsauren UP-I- bzw. UP-III-Lösung (c_{HCl} = 0.3 *N*) wurden in Duranglasröhrchen (innerer Durchmesser: 10 mm; Wandstärke: 1 mm; Länge: 150 mm) auf 4° abgekühlt und mithilfe einer Gasballastpumpe auf einen Druck von $p \leqq 1$ Torr evakuiert. Nach dem Abschmelzen in der Sauerstoffflamme wurden die einzelnen Proben für 4 h bei 200° im Trockenschrank, in Sand eingebettet, decarboxyliert. Anschliessend wurde das entstandene CP-I bzw. CP-III aus der Reaktionslösung nach Zugabe von 0.6 ml 1 *N* NaOH mit 1 ml TBP extrahiert.

Die Rückextraktion erfolgte mit 0.2 ml 5 *N* HCl. Die organische Phase wurde daraufhin in 5 ml Benzol p.A. aufgenommen und nach dem Zentrifugieren abpipettiert. Anschliessend wurde nochmals sukzessive mit 4×5 ml Benzol nachgewaschen, um die salzsäuren CP-I- bzw. CP-III-Lösungen von anhaftenden TBP-Resten vollständig zu befreien. Porphyrine sind unter diesen Bedingungen in Benzol völlig unlöslich, so dass hierbei keine Verluste zu befürchten sind. Die Umsetzung von UP-I bzw. UP-III zu CP-I bzw. CP-III verlief bei Mengen von 1–500 μg unter den angegebenen Bedingungen praktisch vollständig (Ausbeute: A $\cong$ 95 %), was dünnschichtchromatographisch leicht nachzuweisen war.

Extraktionschromatographische Auftrennung der freien Uroporphyrinisomere im TBP–HCl-System. Die zur Durchführung der Isomerentrennung verwendete, weitgehend automatisierte Versuchsanordnung wurde bereits ausführlich an anderer Stelle beschrieben[13,14]. Im Gegensatz zur Trennung von Coproporphyrin–Uroporphyrin[13], die bereits in 1–2 h abgeschlossen ist, werden bei der Auftrennung von UP-I/UP-III, wegen des geringen Trennfaktors ($\alpha = 1.19$), extrem lange Kolonnen ($L_s = 120$ cm) benötigt, wodurch grosse Elutionszeiten $t_{El.}$ resultieren. Als Elutionsmittel wurde stets 1 *N* HCl verwendet; bei der Auftrennung wurde eine Säulentemperatur von $T_s = 30°$ vorgegeben. Für extrem ungünstige UP-I/UP-III-Verhältnisse empfiehlt sich allerdings, die Säulentemperatur auf $T_s = 15°$ zu reduzieren.

Die quantitative Bestimmung der beiden Isomere erfolgte über eine Auswertung des bei der Elution fortlaufend registrierten Transmissionsverlaufes. Im Gegensatz zu einer an anderer Stelle[13] gewählten Definition der Messgrösse wird in der vorliegenden Arbeit der gemessene Transmissionspeak zunächst graphisch digitalisiert (Fig. 5) und die Transmissionsordinaten T_t in die entsprechenden Extinktionswerte E_t umgerechnet (Fig. 6). Als eigentliche, der vorgegebenen Porphyrinmenge $m_{vorg.}$ korrespondierende Messgrösse wird das über den gesamten Peakverlauf von t_1 bis t_2 gehende Extinktionsintegral $N_p = \int_{t_1}^{t_2} E_t \, dt$ bei der Berechnung berücksichtigt. Es ist vorgesehen, diese zunächst noch sehr aufwendige Form der manuellen Auswertung geeigneten, derzeit im Handel erhältlichen Gerätebausteinen zu übertragen (Transmissions–Extinktionskonverter; Digitalintegrator), die ohne Schwierigkeiten in die vorliegende Versuchsanordnung eingebaut werden können[14].

Zur Trennung der Uroporphyrinisomere I und III wird im einzelnen wie folgt vorgegangen: Die in 5–10 ml schwach salzsäurer Lösung (pH 1–3) vorliegenden Isomere werden in den Vorraum der Trennsäule eingefüllt. Bei kleinem Durchfluss D ($D = 15$ ml/h) erfolgt vollständige Extraktion in der obersten Schicht des Füllmaterials ($K_{DB} \geqq 1000$ (Lit. 12)), deren Höhe, je nach der vorliegenden Porphyrinmenge, zwischen 1–10 mm liegt. Anschliessend wird mit 3–5 ml 0.01 *N* HCl nachgewaschen.

Bei der Abtrennung der Hämpräkursoren aus Urin wird folgendermassen verfahren[12,13]: In dem schwach salzsauren Urin (pH 1–3), dessen Volumen, je nach der vorliegenden Porphyrinkonzentration, zwischen 20–200 ml liegt, werden 1–10 g mit TBP imprägniertes Kieselgel aufgeschlämmt, und diese Suspension über eine mit einem Glasfaserfilter belegte Hahn'sche Nutsche (Durchmesser: 50 mm, Porenweite: G 2) abgesaugt. Anschliessend wird das Filtrat noch zweimal über den gleichen Filterboden gegeben und schliesslich mit 0.05 *N* HCl gründlich nachgewaschen. Die durch den Filterboden extrahierten Porphyrine werden nun mit 3×3 ml 5 *N* HCl

sukzessive eluiert, die Lösungen in einem 20 ml Messkölbchen vereinigt und mit Aqua dest. bis zur Marke aufgefüllt. Durch Titration mit 1 *N* NaOH wird die Säurekonzentration der Lösung genau ermittelt. Ein Aliquot hiervon wird mit 1 *N* NaOH auf eine Azidität von $c_{HCl} = 0.01–0.1$ *N* eingestellt und anschliessend zur Auftrennung der Porphyrine auf der Säule, wie vorangehend beschrieben, eingesetzt.

Anschliessend wird die Vorwaschsäule, die mit dem verwendeten Elutionsmittel (1 *N* HCl) bis zum Ausgang gefüllt ist, aufgesetzt und die Elution durch Einschalten der Proportionierpumpe eingeleitet. Die Aufzeichnung des Transmissionsverlaufes durch den Potentiometerschreiber erfolgt kontinuierlich und bedarf keinerlei Wartung während des Versuches. Nach der Auftrennung wird die Menge der einzelnen Hämpräkursoren über eine Auswertung des registrierten Transmissionsverlaufes in der beschriebenen Weise ermittelt.

Dünnschichtchromatographische Auftrennung der freien Porphyrine. Die zu Kontrollzwecken durchgeführten dünnschichtchromatographischen Auftrennungen der freien Porphyrine, insbesondere zur Identifikation der nach der Decarboxylierung erhaltenen Coproporphyrinisomere I und III, wurden nach der an anderer Stelle eingehend beschriebenen Methode[3] vorgenommen. Zu den Trennungen wurden für 2 h bei 120° aktivierte Kieselgelschichten eingesetzt. Als Fliessmittel wurde 2,6-Lutidin–Wasser in der Zusammensetzung 2,6-Lutidin – Aqua dest. (5:3) verwendet, das durch Einstellen eines Becherglases mit 100 ml konz. Ammoniak in die Trennkammer gut vorzusättigen ist.

ERGEBNISSE UND DISKUSSION

Trennung der Uroporphyrinisomere I und III

Die Trennung von Uroporphyrin I und III in freier Form scheiterte bisher vorwiegend daran, dass beide Isomere bei den beschriebenen chromatographischen Methoden in ihrem Verhalten weitgehend übereinstimmten (gleiche R_F-Werte; identische Elutionspositionen), was einerseits in dem Auftreten sehr ungünstiger Trennfaktoren ($\alpha \approx 1$), zum anderen in der sehr ausgeprägten Tendenz zur Aggregatbildung zum Ausdruck kommt.

Voraussetzung für das Gelingen einer solchen Trennung ist zunächst ein deutlich von 1 verschiedener Trennfaktor. Für das vorliegende Isomerenpaar UP-I/UP-III wurde im Batch-Versuch bei $c_{HCl} = 1$ *N* an Kieselgelfilterböden ein statistisch gesicherter Trennfaktor von $\alpha = 1.16 \pm 0.02$ (95 %-Abweichung) erhalten, der im Säulenversuch bei Hostaflon C 2/TBP-Füllung weitgehend bestätigt wurde ($\alpha = 1.19 \pm 0.02$). Es war daher Aufgabe der vorliegenden Untersuchungen, die Versuchsbedingungen bzw. Säulenabmessungen zu ermitteln, die eine Auftrennung und quantitative Bestimmung der beiden Isomeren durchzuführen gestatteten.

Das im folgenden zu beschreibende Auswertverfahren setzt voraus, dass der relative Abstand der beiden Elutionspeaks $\Delta x_p/\overline{T}_{50} \approx 2$ (Δx_p = Abstand der Elutionsmaxima zweier Peaks; $\overline{T}_{50}$ = Arithmetisches Mittel der Halbwertsbreiten $T_{50}{}^a$ und $T_{50}{}^b$ der beiden Einzelpeaks) beträgt[13]. Hierdurch ist eine Bestimmung selbst bei sehr ungünstigen Konzentrationsverhältnissen noch möglich (16 ≧ UP-I/UP-III ≧ 1/16). Eine vollständige Auftrennung der beiden Elutionspeaks, die lange Elutionszeiten $t_{El.}$ erfordert, ist hierbei nicht notwendig. Bei dieser Bestimmung wird weiterhin vorausgesetzt, dass der Verlauf der Elutionspeaks bezüglich Struktur und

Flächenaufteilung bekannt bzw. an Peaks reiner Bezugssubstanzen ermittelt werden kann. Da hierbei Abweichungen von den durch die Normalverteilungsfunktion beschreibbaren Gauss-Peaks auftreten, werden bei dieser Auswertung empirisch ermittelte Peakstruktur- und Peakteilflächenfunktionen für beide Isomere zugrundegelegt[15,16].

Der für reine Bezugssubstanzen UP-I und UP-III unter Versuchsbedingungen erhaltene Peakverlauf ist in Fig. 1 im Wahrscheinlichkeitsnetz dargestellt. Diese

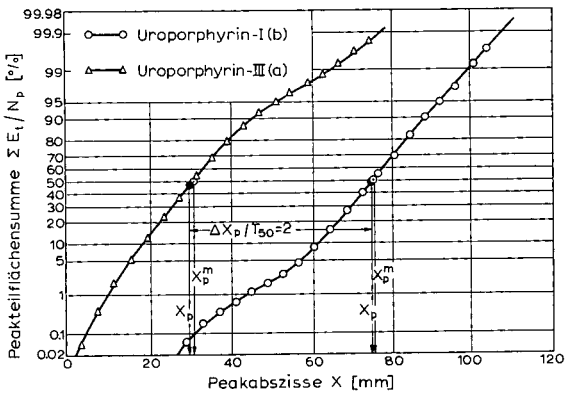


Fig. 1. Auftragung von zwei empirisch erhaltenen, typischen Elutionspeaks von Uroporphyrin-I und -III im Wahrscheinlichkeitsnetz. Beachte die in der rechten Peakflanke von UP-III bzw. linken von UP-I auftretenden Abweichungen von der Form reiner Gausspeaks und die hierdurch verursachten Unterschiede zwischen der mittleren (x_p^m) und wahrscheinlichsten (x_p) Peaklage.

Form der Auftragung lässt besonders deutlich die von der reinen Gauss'schen Peakform auftretenden Abweichungen zum Ausdruck kommen. Die innerhalb enger Grenzen für beide Isomere jeweils erhaltenen numerischen Daten sind in Tabelle II zusammengestellt. Inwieweit die beobachteten Abweichungen von der reinen Gauss'schen Form auf nicht aufgelöste Anteile weiterer Isomere (II, IV) zurückgehen, kann nach dem derzeitigen Stand der Untersuchungen nicht entschieden werden.

Bei der rechnerischen Zerlegung von komplexen Elutionspeaks ist die Kenntnis der Grösse der Überlappungszonen $q_i^{a,r}$ bzw. $q_j^{b,l}$ zur ersten Orientierung häufig von Interesse (Fig. 6). Eine Bestimmung kann für verschiedene Peaklagenabstände $\Delta x_p/\overline{T}_{50}$ und beliebige Intensitätsverhältnisse $N_p{}^a/N_p{}^b$ nach Fig. 2 erfolgen. In dieser Darstellung zu deren Berechnung reine Gauss- und empirische UP-I/UP-III-Peakpaare bei verschiedenen Peaklagenabständen $\Delta x_p/\overline{T}_{50}$ im Wahrscheinlichkeitsnetz ausgewertet wurden (Fig. 1), ist der Anteil $q_j^{b,l}$ von Peak b in der linken Flanke gegen die entsprechenden relativen Peakabszissen $t_i^{a,r}/t_{50}{}^{a,r}$ von Peak a (rechte Flanke) aufgetragen (Fig. 6). Die diesen Peakabszissen korrespondierenden Peakteilflächen $(1-q_i^{a,r})$ von Peak a können für beide Peakpaare ebenfalls Fig. 2 entnommen werden. Zieht man bei zwei nicht aufgelösten Peaks bei einem Peaklagenabstand von $\Delta x_p/\overline{T}_{50}$ eine Trennlinie im Abstand $t_i^{a,r}/t_{50}{}^{a,r}$ von $x_p{}^a$, so kann der Anteil $q_j^{b,l}$, der der restlichen Peakteilfläche $(1-q_i^{a,r})$ überlagert ist, unmittelbar Fig. 2 entnommen werden. So erhält man beispielsweise für das UP-I/UP-III-Peakpaar bei einem Abstand von $\Delta x_p/\overline{T}_{50} = 2$, einem Intensitätsverhältnis $N_p{}^a/N_p{}^b = 1$ und $t_i^{a,r}/t_{50}{}^{a,r} = 1$ den Anteil von Peak b in der Teilfläche von Peak a ($1-q_i^{a,r} = 81.5$ %) zu $q_j^{b,l} = 0.6$ %.

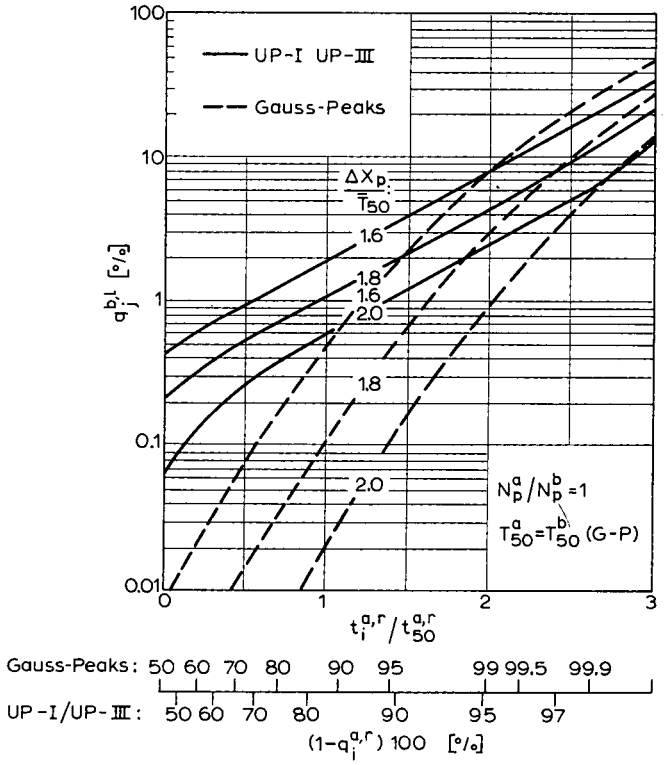


Fig. 2. Darstellung der Peakanteile $q_j^{b,l}$ von Peak b in der Peakteilfläche $(1-q_i^{a,r})$ von Peak a bei verschiedenen Peaklagenabständen ($\Delta x_p/\bar{T}_{50}$ = 1.6, 1.8 und 2.0) in Abhängigkeit von der relativen Peakabszisse $t_i^{a,r}/t_{50}^{a,r}$ für Gauss- und UP-I/UP-III-Peakpaare. Die den $t_i^{a,r}/t_{50}^{a,r}$-Werten entsprechenden Peakteilflächen $(1-q_i^{a,r})$ von Peak a sind ebenfalls für beide Peakpaare im unteren Teil der Abbildung aufgetragen.

Somit beträgt der "Reinheitsgrad" der beiden durch die Trennlinie aufgeteilten Peakteilflächen $\eta_a = q_j^{b,l}/(1-q_i^{a,r}) = 0.74$ % für Peakteilfläche a und $\eta_b = q_i^{a,r}/(1-q_j^{b,l}) = 18.6$ % für Peakteilfläche b. Für ein Gauss-Peakpaar erhält man für die gleichen Bedingungen $\eta_a = q_j^b/(1-q_i^a) = 0.023$ % für Peakteilfläche a und $\eta_b = q_i^a/(1-q_j^b) = 13$% für Peakteilfläche b. Die Darstellung kann in gleicher Weise zur Ermittlung der Überlappungszonen bei Peakpaaren mit beliebigen Intensitätsverhältnissen verwendet werden. Zwischenwerte für die Peaklagenabstände können ebenfalls leicht interpoliert werden.

Diese Bestimmung der Überlappungszonen ist in zweifacher Hinsicht für eine praktische Anwendung von Interesse. So kann bei analytischen Auswertungen der Fehler ermittelt werden, der bei der Zerlegung von nicht aufgelösten Peaks bei einem gegebenen Peaklagenabstand auftritt. Bei präparativen Trennungen kann die Reinheit der zu trennenden Substanzen a und b für nahezu beliebige $t_i^{a,r}/t_{50}^{a,r}$-Werte vorausbestimmt werden, so dass umgekehrt bei einer gegebenen Reinheitsforderung der Zeitpunkt bestimmt werden kann, zu dem das Sammeln der beiden von der Säule eluierten Substanzen begonnen bzw. abgebrochen werden muss.

Die Trennwirkung einer chromatographischen Säule wird für ein gegebenes

Substanzpaar bei definierten Versuchsbedingungen durch die Anzahl der theoretischen Trennböden N charakterisiert. Sie errechnet sich aus dem korrigierten Retentionsvolumen $v_{max.}^{corr.}$ und der Peakabszisse $T_{36.8}$ beim 1/e-ten Teil des Peakmaximums $E_t(x_p)$ nach Lit. 17:

$$N = 8 \left(\frac{v_{max.}^{corr.}}{T_{36.8}} \right)^2 \quad (1)$$

Hieraus erhält man mit der Säulenlänge L_s die Höhe eines theoretischen Bodens Δh zu:

$$\Delta h = \frac{L_s}{N} \quad (2)$$

Die im folgenden zu beschreibende Methode zur Bestimmung der Intensität komplexer Elutionspeaks erfordert im ungünstigsten Fall einen Peaklagenabstand von $\Delta x_p/\overline{T}_{50} \cong 2$. Bei einem Intensitätsverhältnis von $N_p{}^a/N_p{}^b = 1$ erhält man für die Überlappungszonen $q_i{}^{a,r}$ und $q_j{}^{b,l}$ unter der Bedingung:

$$q_i{}^{a,r} = q_j{}^{b,l} \quad (3)$$

aus Fig. 2 durch Interpolation den Wert $q_i{}^{a,r} = q_j{}^{b,l} = 0.036$. Für das vorliegende Isomerenpaar UP-I/UP-III ergibt sich somit nach Lit. 17 mit dem für das TBP–HCl-System erhaltenen Trennfaktor $\alpha = 1.19$ und einem Intensitätsverhältnis $N_p{}^a/N_p{}^b = 1$ die Zahl der erforderlichen Trennböden mit $N = 420$. Ist weiterhin die Höhe eines theoretischen Bodens Δh bekannt, so kann hieraus die Säulenlänge L_s nach Gl. (2) berechnet werden.

Die Trennung der Uroporphyrinisomere I und III mit der beschriebenen Versuchsanordnung wurde unter Bedingungen vorgenommen, wie sie auch weitgehend bei der Auftrennung von Coproporphyrin–Uroporphyrin[13] eingehalten wurden (Säurekonzentration: $c_{HCl} = 1\ N$; Messwellenlänge: $\lambda_{gem.} = 403$ nm; Bandbreite: $\Delta\lambda_B = 10$Å; Säulentemperatur: $T_s = 15$–$30°$; Küvettentemperatur: $T_K = 20°$; Durchfluss: $D = 15$–25 ml/h).

Der Einfluss der Säulenlänge L_s und damit der Anzahl der theoretischen Trennböden auf die Auftrennung eines aus dem Urin eines porphyrie-erkrankten Patienten (Patient U.) isolierten Porphyringemisches geht eindrucksvoll zus den in Fig. 3 dargestellten Beispielen hervor. Die zunehmende Auflösung der beiden Isomere UP-I und UP-III (F 1 und F1a) sowie der übrigen Fraktionen mit wachsender Säulenlänge ist unverkennbar. Die bei dieser Auftrennung erhaltenen charakteristischen Werte für die Elutionszeiten $t_{El.}$ bzw. $t_{El.}^{corr.}$, Peaklagenabstand $\Delta x_p/\overline{T}_{50}$, Trennfaktor α und Zahl der theoretischen Böden N, die mit reinen Bezugssubstanzen UP-I und UP-III bestätigt werden konnten, sind in Tabelle I zusammengestellt. Hieraus kann für das vorliegende Trennsystem die Höhe eines theoretischen Bodens nach Gl. (2) mit $\Delta h = 3.2$ mm berechnet werden. Für die geforderte Auflösung von $\Delta x_p/\overline{T}_{50} = 2$ kann aus den erhaltenen Werten die Zahl der theoretischen Böden zu $N = 425$ ermittelt werden. Dieser Wert stimmt mit dem nach Lit. 17 graphisch erhaltenen gut überein ($N = 420$). Somit errechnet sich die zu der gewünschten Auflösung ($\Delta x_p/\overline{T}_{50} = 2$) unter den vorliegenden Bedingungen ($c_{HCl} = 1\ N$; $T_s = 30°$) erforderliche Säulenlänge zu $L_s = 136$ cm. Bei der praktischen Anwendung wird man jedoch, innerhalb

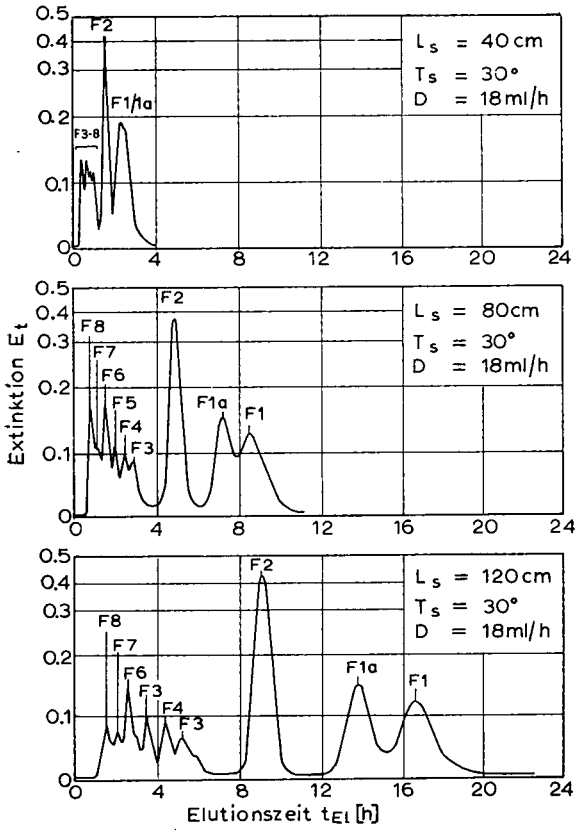


Fig. 3. Extraktionschromatographische Zerlegung eines aus dem Urin eines porphyrie-erkrankten Patienten isolierten komplexen Porphyringemisches unter gleichen Mess- und Versuchsbedingungen, jedoch bei unterschiedlicher Säulenlänge L_s (L_s = 40, 80 und 120 cm). Der Einfluss der Säulenlänge auf die Auflösung ist besonders deutlich bei den Fraktionen F 1/1a (UP-I/UP-III) zu ersehen.

TABELLE I

ZUSAMMENSTELLUNG DER BEI DER AUFTRENNUNG VON UP-I/UP-III MIT SÄULEN UNTERSCHIEDLICHER LÄNGE L_s (L_s = 40, 80 UND 120 cm) ERHALTENEN WERTE FÜR DEN PEAKLAGENABSTAND $\Delta x_p/\overline{T}_{50}$, TRENNFAKTOR α UND ZAHL DER THEORETISCHEN BÖDEN N (FIG. 3)

		L_s (cm)		
		40	80	120
$t_{El.}$ (h)	UP-I	2.65	8.60	16.5
	UP-III	2.40	7.30	13.8
$t_{El.}^{corr.}$ (h)	UP-I	2.35	8.05	15.7
	UP-III	2.10	6.75	13.0
$\Delta x_p/\overline{T}_{50}$		1.10	1.45	1.85
α		(1.12)	1.19	1.21
N		125	250	375

bestimmter Grenzen, die Anpassung an die optimalen Versuchsbedingungen vorzugsweise über die leicht variierbaren Versuchsparameter T_s und c_{HCl} vornehmen[13].

Es muss jedoch nochmals nachdrücklich betont werden, dass eine Auflösung von $\Delta x_p/\overline{T}_{50} = 2$ nur bei extremen Bedingungen erforderlich ist, in den Fällen, in denen eine der beiden Komponenten stark überwiegt (UP-I/UP-III $\geqq$ 10 bzw. $\leqq$ 0.1). Bei günstigeren Konzentrationsverhältnissen kann eine quantitative Bestimmung der beiden Isomere bei wesentlich kleineren Peaklagenabständen durchgeführt werden, ohne dass dadurch die Genauigkeit merklich eingeschränkt würde. So können Isomerengemische bei einem Mengenverhältnis von UP-I/UP-III $\approx$ 1 noch bei einem Peaklagenabstand von $\Delta x_p/\overline{T}_{50} = 1.5$ zerlegt und quantitativ bestimmt werden. Hierdurch kann der zur Durchführung einer Auftrennung erforderliche Zeitaufwand ganz erheblich reduziert werden (Fig. 3).

Identifikation der Uroporphyrinisomere I und III

Bei einem Vergleich der dünnschichtchromatographischen (vgl. Fig. 2, Lit. 12) und extraktionschromatographischen (Fig. 3) Auftrennung des aus dem Urin von Patient U. isolierten Porphyringemisches fällt auf, dass die beiden intensivsten Fraktionen auf der DC-Platte (f 1,2: Fig. 4) bei der EC-Trennung deutlich in drei Peaks unterschiedlicher Intensität aufspalten (F 1,1a und 2: Fig. 3). Die Auftrennung der übrigen Fraktionen (DC-Technik: f 3–9; EC-Technik: F 3–8), über deren Identifizierung an anderer Stelle noch ausführlich berichtet werden soll[14], dagegen stimmt in beiden Fällen weitgehend überein. Es wurde daher zunächst vermutet[12], dass es sich bei den dünnschichtchromatographisch mit niedrigen R_F-Werten erhaltenen Fraktionen f 1,2 ($R_F = 0.02$–0.04) um Uroporphyrinisomere handelt, die extraktionschromatographisch weiter zerlegt werden können. Zur Identifizierung von f 1,2 bzw. F 1,1a und 2 wurden die folgenden Untersuchungen durchgeführt:

Bestimmung der Peaklage λ_p der Soretbande. Die Peaklage der Soretbande λ_p ist neben Schmelzpunkt, R_F-Wert, Salzsäurezahl, Verteilungskoeffizient u.a. ein absolut zuverlässiges Kriterium für die Identität eines Hämpräkursors. Da Isomere gleiche λ_p-Werte aufweisen und zwischen der Anzahl von Carboxylgruppen pro Molekül n und λ_p eine direkte Abhängigkeit besteht[14], kann somit über eine Bestimmung dieser Grösse eine weitgehend eindeutige Einordnung des Hämpräkursors erfolgen. Es ist überraschend festzustellen, dass über diese Möglichkeit der Identifizierung von Porphyrinen bisher in der Literatur kaum berichtet wurde, obwohl die Methode durch ihre hohe Empfindlichkeit und Eindeutigkeit anderen gebräuchlichen Nachweisreaktionen zumindest ebenbürtig ist.

Zu dieser Bestimmung wird der Absorptionsverlauf der Porphyrinlösung bei definierter HCl-Konzentration im Bereich von 380–450 nm spektralphotometrisch bei kleiner Spalt- bzw. Bandbreite gemessen. Durch graphische Differentiation der Soretbande zwischen 390–410 nm erhält man mit hoher Genauigkeit die gesuchte Peaklage λ_p[3,15,16]. Die Bestimmung kann noch selbst mit Mengen von 0.2 μg des Hämpräkursors durchgeführt werden. Auf den Einfluss der Salzsäurekonzentration c_{HCl} auf Peaklage und spezifische Extinktion ist streng zu achten[13].

Im vorliegenden Fall wurden für die einzelnen Fraktionen bei $c_{HCl} = 1\ N$ die folgenden Werte für die Peaklage der Soretbande λ_p erhalten: f 1 sowie F 1, 1a: $\lambda_p = 405.8 \pm 0.1$ nm; f 2 sowie F 2: $\lambda_p = 404.7 \pm 0.1$ nm. Hieraus folgt, dass f 1 bei der extraktionschromatographischen Trennung in zwei Uroporphyrinisomere auf-

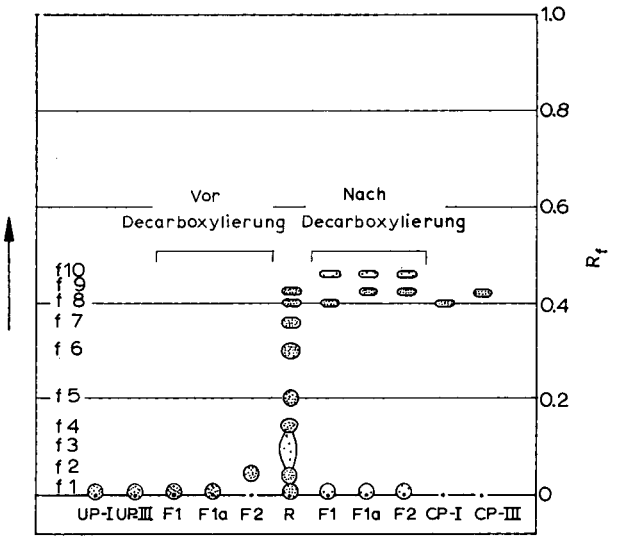


Fig. 4. Dünnschichtchromatographische Auftrennung der extraktionschromatographisch isolierten Fraktionen F 1, 1a und 2, vor und nach der Decarboxylierung. Identifikation durch Vergleich der R_F-Werte mit den reiner Bezugssubstanzen. Die Punktdichte der DC-Flecken widerspiegelt näherungsweise die relative Porphyrinkonzentration. R ist das aus dem Urin von Patient U. isolierte Porphyringemisch (Fig. 2, Lit. 12).

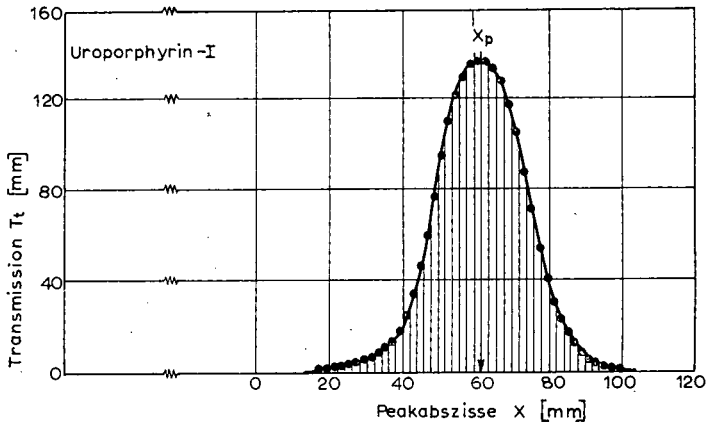


Fig. 5. Zerlegung eines bei der Registrierung erhaltenen typischen Transmissionspeaks von Uroporphyrin-I und Ermittlung der den jeweiligen Peakabszissen x entsprechenden Ordinatenwerte T_t ("graphische Digitalisierung").

spaltet (identische λ_p-Werte), die dünnschichtchromatographisch nicht weiter aufgetrennt werden können (Fig. 4). Aus dem für f 2 sowie F 2 erhaltenen λ_p-Wert kann in Verbindung mit anderen Kriterien bzw. weiteren Umsetzungen[14] geschlossen werden, dass es sich um ein Heptacarboxylporphyrin der Isomerenreihe III handelt (7-III).

Bestimmung der Lage x_p der Elutionspeaks. Anstelle der in der Literatur bisher gebräuchlichen Salzsäurezahl zur Charakterisierung der abgestuften Affinität der einzelnen Porphyrine gegenüber dem verwendeten Extraktionsmittel wird in der vorliegenden Arbeit der Verteilungskoeffizient K_{DB} verwendet, der bei der extraktionschromatographischen Säulentrennung die Lage der Elutionsmaxima x_p bestimmt. Aus den im Batch-Versuch an Kieselgelfilterböden erhaltenen K_{DB}-Werten für UP-I und UP-III (K_{DB}(UP-I) = 58; K_{DB}(UP-III) = 50, jeweils für $c_{HCl} = 1\ N$) folgt, dass UP-I die grössere Elutionszeit $t_{El.}$ aufweisen, somit F 1 mit UP-I und F 1a mit UP-III identisch sein sollten. Die Annahme wurde wie folgt bestätigt: Fügt man zu dem aufzutrennenden Porphyringemisch (Patient U.) vorher dosierte Mengen der reinen Bezugssubstanzen von UP-I und UP-III hinzu, so kann eine adäquate Zunahme der Peakfläche N_p der jeweiligen Fraktion beobachtet werden. Eine Änderung der Peakstruktur bei dieser Aufstockung, kontrolliert über die Peakhalbwertsbreite T_{50}, war weder bei UP-I noch UP-III festzustellen, wodurch die Identität der beiden Fraktionen als nachgewiesen angesehen wurde.

Dünnschichtchromatographische Auftrennung vor und nach der Decarboxylierung. Die derzeit wohl zuverlässigste Methode zur Identifikation der Uroporphyrinisomere UP-I und UP-III besteht in der Überführung in die entsprechenden Coproporphyrinisomere CP-I und CP-III durch Decarboxylierung, die nun ihrerseits dünnschichtchromatographisch aufgetrennt und als solche identifiziert werden können.

Hierzu werden die Fraktionen F1, 1a und 2 nach der von EDMONDSON UND SCHWARTZ beschriebenen Methode[10] umgesetzt, und die Reaktionsprodukte mit den reinen Bezugssubstanzen CP-I und CP-III dünnschichtchromatographisch auf der gleichen Platte entwickelt. Das Ergebnis einer solchen Auftrennung ist aus Fig. 4 zu ersehen.

Ein Vergleich der R_F-Werte beweist, dass bei der Decarboxylierung aus F 1 CP-I und aus F 1a und F 2 CP-III entsteht. Hierdurch ist die Zugehörigkeit von F 1 bzw. F 1a und F 2 zur Isomerenreihe I bzw. III eindeutig sichergestellt, da für CP-II und CP-IV andere R_F-Werte erwartet werden[3]. Eine Bestimmung der Peaklage der Soretbande λ_p der decarboxylierten Hämpräkursoren ergab ebenfalls den für Coproporphyrin in $c_{HCl} = 1\ N$ zu erwartenden Wert (λ_p = 401.3 nm). Weiterhin war auffallend, dass nach der Decarboxylierung von F 1, 1a und 2 eine Substanz in der CP-II-Position auftrat (f 10, Fig. 4), die in dem ursprünglichen Porphyringemisch nicht oder nur in sehr geringer Konzentration vorlag. Da jedoch der ermittelte λ_p-Wert deutlich von dem bei CP-Isomeren zu erwartenden abweicht (λ_p = 402.2 und 403.0 nm), muss bezweifelt werden, ob es sich im Falle von f 10 um ein Coproporphyrinisomer handelt[14].

Quantitative Bestimmung der Uroporphyrinisomere I und III

Mit der beschriebenen Versuchsanordnung können Uroporphyrinisomere in freier Form nicht nur aufgetrennt sondern darüber hinaus in einem Arbeitsgang auch quantitativ bestimmt werden. Hierzu wird der mit einem Potentiometerschreiber kontinuierlich aufgezeichnete Verlauf der Transmission T_t zunächst graphisch digitalisiert (Fig. 5), und die erhaltenen Transmissionsordinaten in die entsprechenden Extinktionswerte E_t transformiert (Fig. 6). Aus der über die gesamte Peakfläche integrierten Extinktion E_t erhält man die der vorgegebenen bzw. vorliegenden Porphyrinmenge $m_{vorg.}$ korrespondierende Messgrösse N_p:

$$N_p = \int_{t1}^{t2} E_t \, dt \tag{4}$$

Eine Ermittlung der den einzelnen Fraktionen entsprechenden Peakflächen N_p wird häufig dadurch beeinträchtigt, dass die Auflösung der Elutionspeaks unzureichend ist bzw. dass zur vollständigen Auflösung sehr lange Säulen und damit grosse Elutionszeiten $t_{El.}$ erforderlich sind (Fig. 3). Zur Reduzierung des Zeitaufwandes bei der Analyse von komplexen Porphyringemischen wird im folgenden ein Verfahren beschrieben, das eine Zerlegung der Elutionspeaks selbst bei mässiger Peaküberlagerung ($\Delta x_p/\overline{T}_{50} = 1.5$–$2.0$, je nach Mengenverhältnis $N_p{}^a/N_p{}^b$) noch durchzuführen gestattet. Diese Methode, zur Zerlegung komplexer γ-Spektren entwickelt[15,16], berücksichtigt exakt dem empirisch erhaltenen Transmissions- bzw. Extinktionsverlauf der Elutionspeaks, der von dem reiner Gauss-Peaks oft merklich abweicht. Hiermit können allgemein komplexe Gauss- bzw. Pseudo-Gauss-Peaks unter der Voraussetzung zerlegt werden, dass:

(1) die Peakstruktur der beiden Einzelpeaks genau bekannt ist,
(2) eine der beiden Peaklagen $x_p{}^a$ bzw. $x_p{}^b$ genau bestimmt werden kann,
(3) das Intensitätsverhältnis $N_p{}^a/N_p{}^b$ innerhalb der Grenzen $16 \geqq N_p{}^a/N_p{}^b \geqq 0.06$ liegt.

Zur Durchführung der quantitativen Bestimmung ist stets eine Kalibrierung der Anordnung unter den jeweiligen Versuchs- bzw. Messbedingungen mit reinen Bezugssubstanzen erforderlich.

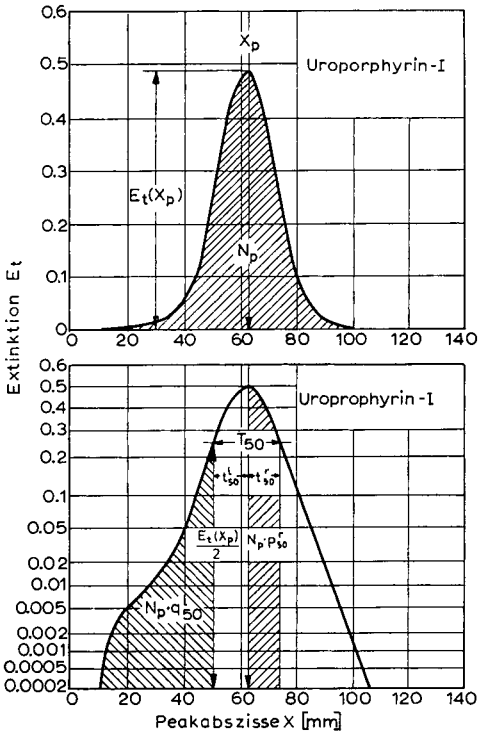


Fig. 6. Auftragung der nach Digitalisierung und Umrechnung erhaltenen Extinktionswerte E_t (Fig. 5) im bilinearen Masstab (obere Darstellung) bzw. im Häufigkeitsnetz (untere Darstellung).

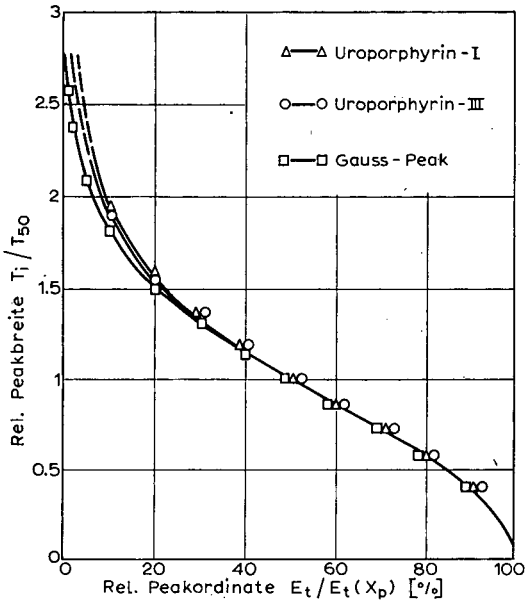


Fig. 7. Verlauf der relativen, auf die Peakhalbwertsbreite T_{50} bezogene Peakbreite T_i/T_{50} in Abhängigkeit von der relativen Peakordinate $E_t/E_t(x_p)$ $(= u_i)$ für die beiden Uroporphyrinisomere I und III sowie für reine Gauss-Peaks.

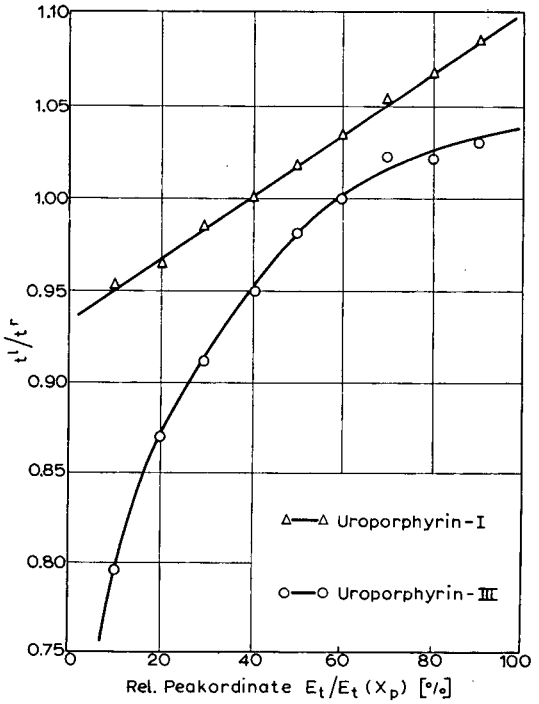


Fig. 8. Verlauf der Quotienten der Peakabszissen t_i^l/t_i^r in Abhängigkeit von der relativen Peakordinate u_i für die beiden Uroporphyrinisomere I und III.

Bei der Ermittlung der Peakflächen $N_p{}^a$ bzw. $N_p{}^b$ von zwei sich überlagernden Elutionspeaks wird ein bestimmter Peakabschnitt, der von Anteilen des Nachbarpeaks frei sein muss, ausgewertet. Die zwischen den diesen Abschnitt begrenzenden Peakabszissen $t_i{}^{r,l}$ bzw. T_i (Fig. 6) und den Peakteilflächen $N_p \cdot q_i$ bzw. $N_p \cdot p_i$ vorliegende Beziehung ("Peakteilflächenfunktion") kann für reine Gauss'sche Peaks berechnet (Gl. 12, 13) bzw. für Pseudo-Gauss-Peaks empirisch ermittelt werden. Sie gestatten die Umrechnung der Peakteilflächen auf die gesamte Peakfläche N_p.

Für reine, durch die Normalverteilungsfunktion beschreibbare Gauss-Peaks:

$$E_t = \frac{N_p}{\sigma\sqrt{(2\pi)}} \exp\left(-\frac{(x-x_p)^2}{2\sigma^2}\right) \tag{5a}$$

mit $x - x_p = t$

$$E_t = \frac{N_p}{\sigma\sqrt{(2\pi)}} \exp\left(-\frac{t^2}{2\sigma^2}\right) \tag{5b}$$

lässt sich zeigen, dass zwischen der relativen, auf das Peakmaximum $E_t(x_p)$ bezogenen Peakordinate $E_t/E_t(x_p)$ $(= u_i)$ und der auf die Peakbreite bei $\frac{1}{2}E_t(x_p)$ bezogenen relativen Peakabszissen T_i/T_{50} der folgende Zusammenhang besteht[15,16]:

$$\frac{T_i}{T_{50}} = \sqrt{\frac{-\ln u_i}{\ln 2}} \tag{6}$$

Eine an unter Versuchsbedingungen erhaltenen Elutionspeaks durchgeführte Analyse der Peakstruktur ergab, dass auch empirische Peaks, unabhängig von der vorliegenden Intensität N_p und Peaklage x_p, durch eine einzige "Peakstrukturfunktion" beschrieben werden können, die alle individuellen Abweichungen von der rein Gauss'schen Peakform erfasst (Fig. 7, Tabelle II). Hierbei wird für UP-I und UP-III, wie nach Fig. 1 zu erwarten, ein unterschiedlicher Verlauf beobachtet. Die zwischen rechter und linker Peakflanke bestehenden Unterschiede bzw. die hierdurch bedingte Peakasymmetrie macht eine Unterscheidung zwischen rechter und linker Peakabszisse, $t_i{}^l$ und $t_i{}^r$, erforderlich. Der Verlauf des Quotienten der Peakabszissen $t_i{}^l/t_i{}^r$ ist in Abhängigkeit von der relativen Peakhöhe $E_t/E_t(x_p)$ in Fig. 8 aufgetragen.

Mit den derart empirisch ermittelten Funktionen:

$$\frac{T_i}{T_{50}} = \mathrm{f}(u_i) \tag{7}$$

$$\frac{t_i^l}{t_i^r} = \mathrm{f}(u_i) \tag{8}$$

kann somit der normierte Verlauf der Elutionspeaks, unabhängig von Intensität und Peaklage, exakt beschrieben werden.

In Analogie zur Normierung der Peakstruktur lässt sich zeigen, dass auch bei der schrittweisen Integration der Elutionspeaks mit den durch die relative Peakhöhe u_i über die entsprechenden Peakabszissen $t_i{}^{r,l}$ bestimmten Integrationsgrenzen die erhaltenen Peakteilflächen $N_p \cdot q_i$ bzw. $N_p \cdot p_i$ (Fig. 6) jeweils bestimmten Anteilen der gesamten Peakfläche N_p entsprechen, die erhaltenen p_i- bzw. q_i-Werte somit ebenfalls von N_p, x_p und T_{50} unabhängig sind.

Der Berechnung der Peakflächenabschnitte q_i für reine Gauss-Peaks liegt der folgende Ansatz zugrunde:

$$q_i = \frac{1}{\sigma\sqrt{2\pi}} \int_t^\infty \exp\left(-\frac{t^2}{2\sigma^2}\right) \mathrm{d}t \tag{9}$$

Durch Einsetzen der Variablen v:

$$v = \frac{t}{\sigma\sqrt{2}}$$

und entsprechende Umformung erhält man bei der von den Flanken zur Peakmitte hin bis zu den jeweiligen relativen Peakordinaten u_i durchgeführten Integration die Peakanteile q_i in der Form:

$$q_i = \frac{1}{2}\left(1 - \frac{2}{\sqrt{\pi}} \int_0^v \exp(-v^2)\, \mathrm{d}v\right) \tag{10}$$

Zur numerischen Auswertung wird das Fehlerintegral in eine Potenzreihe entwickelt, woraus die gesuchten q_i-Werte durch gliedweise Integration der Reihe erhalten werden:

$$q_i = \frac{1}{2}\left(1 - \frac{2}{\sqrt{\pi}} \int_0^v \left(1 - \frac{v^2}{1!} + \frac{v^4}{2!} - \frac{v^6}{3!} - \ldots + \ldots\right) \mathrm{d}v\right) \tag{11}$$

Unter Berücksichtigung der Integrationsgrenzen u_i erhält man somit:

$$q_i = \frac{1}{2}\left\{1 - \frac{2}{\sqrt{\pi}}\left[\sqrt{-\ln u_i} - \frac{1}{3}(\sqrt{-\ln u_i})^3 + \frac{1}{10}(\sqrt{-\ln u_i})^5 - \ldots\right]\right\} \tag{12}$$

Bei der schrittweisen Integration von der Peakmitte x_p zum Flankenauslauf hin erhält man für die komplementären p_i-Werte den Ausdruck:

$$p_i = \frac{1}{\sqrt{\pi}}\left[\sqrt{-\ln u_i} - \frac{1}{3}(\sqrt{-\ln u_i})^3 + \frac{1}{10}(\sqrt{-\ln u_i})^5 - \ldots\right] \tag{13}$$

Eine an 23 Elutionspeaks durchgeführte Auswertung ergab, dass auch bei empirischen Peaks die erhaltenen q_i- bzw. p_i-Werte von T_{50}, σ und N_p unabhängig sind. Die innerhalb enger Grenzen für UP-I und UP-III erhaltenen Funktionen ("Peakteilflächenfunktionen"), die wegen der vorliegenden Peakasymmetrie für die rechte und linke Peakflanke jeweils getrennt ermittelt werden müssen:

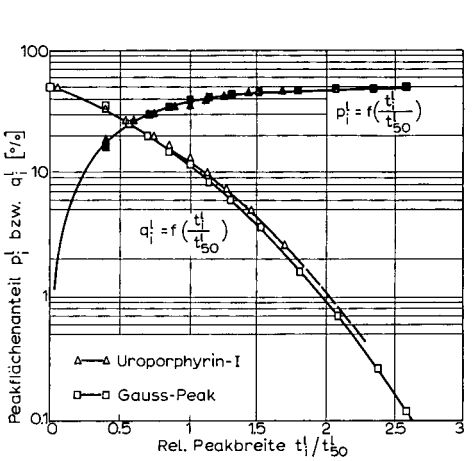
$$p_i^l = \mathrm{f}(u_i) \qquad p_i^r = \mathrm{f}(u_i) \tag{14a}$$

$$q_i^l = \mathrm{f}(u_i) \qquad q_i^r = \mathrm{f}(u_i) \tag{14b}$$

sind numerisch ausgewertet nochmals mit den zugehörigen doppelten, mittleren Abweichungen der Mittelwerte (95 %-Fehler) in Tabelle II zusammengestellt.

Zum Vergleich sind in Fig. 9 die für UP-I in der linken Peakflanke erhaltenen Peakflächenabschnitte p_i^l bzw. q_i^l gegen die relativen Peakordinaten u_i aufgetragen und den für eine reine Gauss-Flanke gültigen Werten gegenübergestellt.

Eine Auftragung der Quotienten p_i^l/p_i^r in Abhängigkeit von der relativen Peakhöhe u_i (Fig. 10) bringt die bei beiden Elutionspeaks (UP-I und UP-III) vorliegende Asymmetrie einerseits, sowie die zwischen UP-I und UP-III bestehenden strukturellen Verschiedenheiten andererseits besonders deutlich zum Ausdruck.



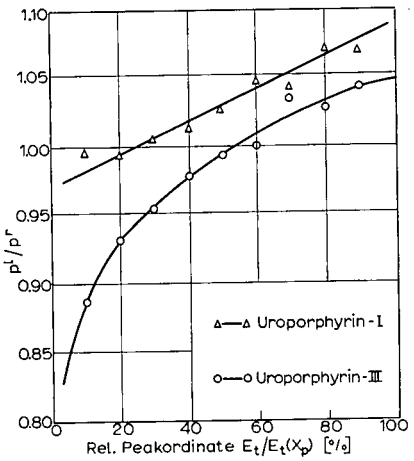


Fig. 9. Abhängigkeit der Peakflächenanteile $p_i{}^l$ bzw. $q_t{}^l$ von der relativen Peakbreite $t_i{}^l/t_{50}{}^l$ für die linke Flanke von Uroporphyrin-I sowie für einen reinen Gauss-Peak.

Fig. 10. Verlauf des Quotienten der Peakteilflächenanteile $p_t{}^l/p_t{}^r$ in Abhängigkeit von der relativen Peakordinate u_i für die beiden Uroporphyrinisomere I und III.

TABELLE II

ZUSAMMENSTELLUNG DER FÜR EINEN REINEN GAUSS-PEAK BZW. EMPIRISCHE ELUTIONSPEAKS VON UROPORPHYRIN-I (OBERE ZEILE) UND UROPORPHYRIN-III (UNTERE ZEILE) FÜR RECHTE (r) UND LINKE (l) PEAKFLANKE ERHALTENEN RELATIVEN PEAKBREITEN $(T_i/T_{50})_g$ BZW. T_i/T_{50}, QUOTIENTEN DER PEAKABSZISSEN $t_i{}^l/t_i{}^r$ SOWIE DER PEAKFLACHENABSCHNITTE $p_i{}^g$ BZW. $p_i{}^r$ UND $p_i{}^l$ IN ABHÄNGIGKEIT VON DER RELATIVEN PEAKORDINATE u_i

u_i	T_i/T_{50}	$(T_i/T_{50})_g$	$t_i{}^l/t_i{}^r$	$p_i{}^l$ (%)	$p_i{}^r$ (%)	$p_i{}^g$ (%)
0.10	1.92_7	1.823	0.95_{16}	47.41 ± 0.72	47.59 ± 1.15	48.40
	1.90_7		0.794_9	45.13 ± 1.03	50.91 ± 1.04	
0.20	1.55_7	1.523	0.963_4	45.03 ± 0.83	45.34 ± 1.02	46.36
	1.55_0		0.868_9	43.55 ± 0.88	46.79 ± 1.24	
0.30	1.32_7	1.318	0.984_8	42.76 ± 0.93	42.57 ± 0.98	43.96
	1.32_3		0.911_1	41.58 ± 0.96	43.63 ± 1.37	
0.40	1.15_4	1.150	0.999_1	40.20 ± 1.00	39.67 ± 1.02	41.21
	1.14_7		0.949_0	39.10 ± 1.06	40.05 ± 1.32	
0.50	1.000	1.000	1.01_8	37.33 ± 1.29	36.39 ± 1.05	38.05
	1.000		0.981_4	36.65 ± 0.92	36.99 ± 1.36	
0.60	0.864_0	0.8582	1.03_3	33.96 ± 1.64	32.54 ± 0.82	34.39
	0.856_0		1.00_9	33.38 ± 0.96	33.11 ± 1.29	
0.70	0.731_9	0 7170	1.05_3	30.39 ± 1.36	29.11 ± 1.25	30.08
	0.716_9		1.02_6	29.51 ± 1.04	28.50 ± 1.18	
0.80	0.594_2	0.5675	1.06_7	25.71 ± 1.30	24.13 ± 1.28	24.80
	0.571_3		1.02_2	24.45 ± 1.03	23.81 ± 0.76	
0.90	0.417_6	0.3900	1.08_6	18.94 ± 1.28	17.77 ± 0.75	17.69
	0.386_5		1.03_0	17.34 ± 0.96	16.68 ± 1.07	

Bei der Zerlegung von sich überlagernden Elutionspeaks ist Voraussetzung, dass die Peaklage x_p zumindest eines der beiden Peaks ermittelt werden kann. Eine solche Bestimmung kann mit den an anderer Stelle beschriebenen Methoden, der sog. Differenzen- bzw. Quotientenmethode (Differentiation des normalen bzw. logarithmierten Peakverlaufes), mit der erforderlichen Genauigkeit vorgenommen werden[15,16]. Die Wahl der Methode hängt u.a. davon ab, welcher Peaklagenabstand $\Delta x_p/\overline{T}_{50}$ und welches Intensitätsverhältnis $N_p{}^a/N_p{}^b$ im jeweiligen Falle vorliegt.

Zur Verdeutlichung des Einflusses dieser Parameter sind in Fig. 11 sechs Beispiele von unter Versuchsbedingungen erhaltenen komplexen Elutionspeaks zusammengestellt, die, im Häufigkeitsnetz aufgetragen[15], das Ausmass der Peaküberlappung bei den verschiedenen $\Delta x_p/\overline{T}_{50}$- bzw. $N_p{}^a/N_p{}^b$-Werten eindrucksvoll zum Ausdruck bringen.

Während eine Bestimmung der Peaklage x_p mit der Differenzenmethode nur in solchen Fällen durchgeführt werden kann, bei denen das auszuwertende Peakmaximum weitgehend frei von Anteilen des Nachbarpeaks ist (Fig. 11: $N_p{}^a/N_p{}^b = 1{:}\Delta x_p/\overline{T}_{50} = 1.5$–$2.0$; $N_p{}^a/N_p{}^b = 10{:}\Delta x_p/\overline{T}_{50} = 2.0$), kann die Quotientenmethode auch bei komplexen Peakpaaren mit wesentlich kleineren Peaklagenabständen ($\Delta x_p/\overline{T}_{50} \approx 1$) und ungünstigeren Intensitätsverhältnissen ($N_p{}^a/N_p{}^b \approx 10$) mit Erfolg eingesetzt werden[15,16]. Die letztere Methode setzt jedoch, im Gegensatz zur Differenzenmethode, voraus, dass der zur Auswertung gelangende Flankenauslauf exakt durch die Normalverteilungsfunktion beschrieben bzw. im Häufigkeitsnetz

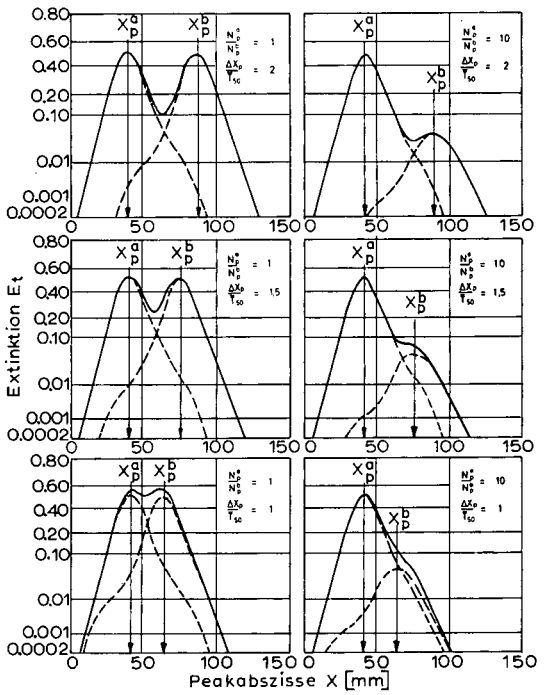


Fig. 11. Zusammenstellung von einigen typischen komplexen Peakpaaren von UP-I/UP-III für verschiedene Peaklagenabstände $\Delta x_p/\overline{T}_{50}$ ($\Delta x_p/\overline{T}_{50}$ = 1.0, 1.5 und 2.0) und Mengenverhältnisse UP-III/UP-I (UP-III/UP-I = 1 und 10).

entsprechend korrigiert werden kann. Diese Bedingung ist jedoch im Falle der Elutionspeaks von UP-I und UP-III nicht oder nur in unzureichendem Umfange erfüllt (vgl. Fig. 1, 6, 8, 10), so dass in der vorliegenden Untersuchung lediglich die Differenzenmethode zur Bestimmung der Peaklage x_p zur Anwendung gelangt.

Bei der Ermittlung der Peaklagen mit verschiedenen Methoden ist zu beachten, dass, bedingt durch die den empirischen Peaks meist zugrundeliegende asymmetrische Struktur, zwischen mittlerer (x_p^m) und wahrscheinlicher (x_p) Peaklage unterschieden werden muss. Während bei einer Auswertung im Wahrscheinlichkeitsnetz x_p^m-Werte erhalten werden (Fig. 1), erhält man mit der Quotienten- und Differenzenmethode stets die auf das Peakmaximum bezogenen Werte x_p-Werte.

Bei der quantitatieven Bestimmung der beiden Uroporphyrinisomere I und III wurde wie folgt vorgegangen: Der analog aufgezeichnete Transmissionsverlauf wurde zunächst digitalisiert (Fig. 5) und in die entsprechenden Extinktionswerte E_t umgerechnet. Durch graphische Differentiation des um das Peakmaximum liegenden Extinktionsverlaufes erhält man für $dE_t/dx = 0$ die gesuchte Peaklage x_p mit hoher Genauigkeit[3,15,16]. Da bei konstanten Versuchsbedingungen die Halbwertsbreite T_{50} mit Bezugssubstanzen leicht ermittelt, bzw. bei variablen Versuchsbedingungen die zwischen x_p und T_{50} bestehende Abhängigkeit ebenfalls mit reinem UP-I und UP-III auf einfache Weise erhalten werden kann, lässt sich somit aus der Peaklage x_p, der Halbwertsbreite T_{50} und den empirisch ermittelten Peakstrukturfunktionen (Gl.7, 8) der Peakverlauf bei Proben unbekannter Zusammensetzung exakt darstellen.

Die erhaltenen E_t-Werte werden nun im Häufigkeitsnetz aufgetragen und über das Peakmaximum hinausgehend fortlaufend summiert (Fig. 12). Hierbei ist zu beachten, dass die einzelnen Werte die Mitte der bei der Digitalisierung erhaltenen jeweiligen Kanäle bzw. Klassen darstellen, deren Bereich durch die folgenden Grenzen $t \pm \frac{1}{2}$ bzw. $x \pm \frac{1}{2}$ festgelegt ist.

Aus den empirisch ermittelten Beziehungen (Gl. 7, 8) erhält man die Peakabszissen t_i^r, die den jeweiligen relativen Peakordinaten u_i in der rechten Peakflanke von UP-I entsprechen. Diese (t_i^r) stellen nun wiederum auf der in der gleichen Dar-

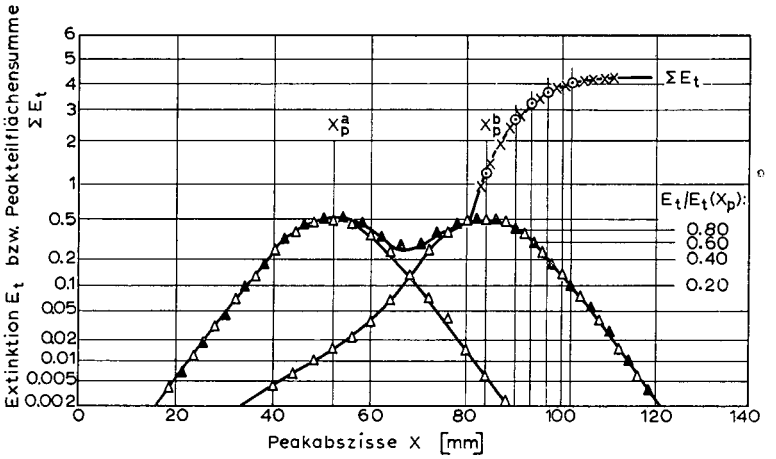


Fig. 12. Bestimmung der Fläche von Peak b (UP-I) über eine Ermittlung der Peakteilflächen $N_p^b \cdot p_i^l$ bei verschiedenen relativen Peakordinaten u_i bzw. den entsprechenden Peakabszissen t_i^r/t_{50}^r.

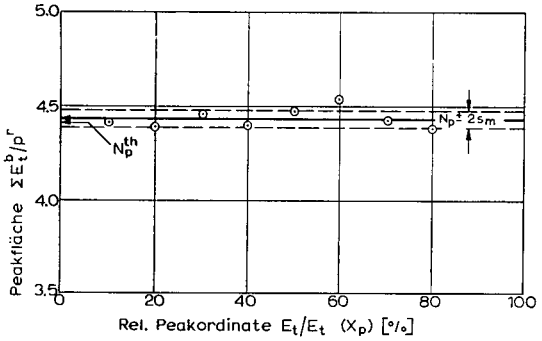


Fig. 13. Ermittlung der Peakfläche $N_p{}^b$ durch Extrapolation der bei verschiedenen Peakordinaten u_i erhaltenen Werte (Fig. 12) auf $u_i = 0$.

stellung eingezeichneten Summenkurve ΣE_t die Integrationsgrenzen dar, die Peakteilflächen $N_p{}^b \cdot p_i{}^r$, die ihrerseits über die Peakstrukturfunktion (Gl. 14a) mit der gesamten Peakfläche $N_p{}^b$ in einem bekannten Verhältnis $p_i{}^r$ stehen, einschliessen. Aus den bei beliebigen u_i- bzw. $t_i{}^r$-Werten erhaltenen Peakteilflächen $\Sigma E_{t,i}$ kann somit mit den zugehörigen $p_i{}^r$-Werten die Peakfläche $N_p{}^b$ über die Definitionsgleichung:

$$N_p^b = \frac{\Sigma E_{t,i}}{p_i{}^r} \tag{16}$$

berechnet werden. Diese Form der Bestimmung von $N_p{}^b$ schliesst Auswertefehler weitgehend aus und gestattet, die Peakfläche innerhalb einer relativ kleinen Fehlerspanne festzulegen. Eine Auftragung der bei verschiedenen relativen Peakordinaten u_i erhaltenen $N_p{}^b$-Werte (Fig. 13), die bei der Auswertung einer reinen Peakflanke eine der Abszisse parallel laufende Gerade ergibt, erlaubt Rückschlüsse auf ev. vorhandene bzw. nicht korrigierte Anteile der Nachbarpeaks[15,16].

In analoger Weise kann grundsätzlich die Bestimmung der Peakfläche von Peak a, $N_p{}^a$, vorgenommen werden. Diese kann jedoch in einfacherer Weise aus der Peakfläche des komplexen Peakpaares $N_p{}^c$ und dem ermittelten $N_p{}^b$-Wert nach:

$$N_p^c = N_p^a + N_p^b \tag{17}$$

erhalten werden.

Die quantitative Bestimmung der Porphyrine über eine Auswertung der Fläche der Elutionspeaks ist in der beschriebenen Weise noch recht aufwendig. Bei Verwendung eines Transmissions–Extinktionswandlers, der bei der Durchführung dieser Untersuchungen nicht zur Verfügung stand, kann der zur Auswertung erforderliche Zeitaufwand jedoch ganz wesentlich reduziert werden, besonders dann, wenn die Ermittlung der Peakteilflächen mit einem Disc-Integrator vorgenommen wird. Über diese Form der Auswertung von in Transmissionseinheiten aufgezeichneten Elutionspeaks wurde bereits ausführlich an anderer Stelle berichtet[13]. Die geplante Erweiterung der beschriebenen Versuchsanordnung[14] gestattet auch die Auswertung von Elutionschromatogrammen im Routinebetrieb.

Zur Prüfung der unter Versuchsbedingungen erreichbaren Empfindlichkeit und Genauigkeit wurden Mischungen der beiden Uroporphyrinisomere in bekannter Zusammensetzung unter geeigneten Versuchsbedingungen aufgetrennt und hierbei

jeweils UP-I in der beschriebenen Weise quantitativ bestimmt. Während der UP-III-Anteil jeweils konstant ($m_{vorg.} = 28.7$ μg) blieb, variierte die UP-I-Menge im Bereich von 1.73–27.6 μg. Die zwischen der vorgegebenen UP-I-Menge $m_{vorg.}$ und der Fläche der Elutionspeaks N_p erhaltene Abhängigkeit ist in Fig. 14 dargestellt. Aus dieser Auftragung geht hervor, dass bei einem Peaklagenabstand von $\Delta x_p/\overline{T}_{50} \cong 2$ eine Bestimmung der beiden Isomere noch durchgeführt werden kann, selbst wenn das Isomerenverhältnis UP-III/UP-I = 16 beträgt. Die untere Grenze, bei der unter den vorliegenden Versuchsbedingungen eine Bestimmung noch möglich ist, wird bei *ca.* 1 μg angenommen. Die in Fig. 14 ebenfalls eingezeichneten spezifischen Extinktionsflächen $N_p/m_{vorg.}$, die mit den der reinen Substanz innerhalb der Fehlerspanne

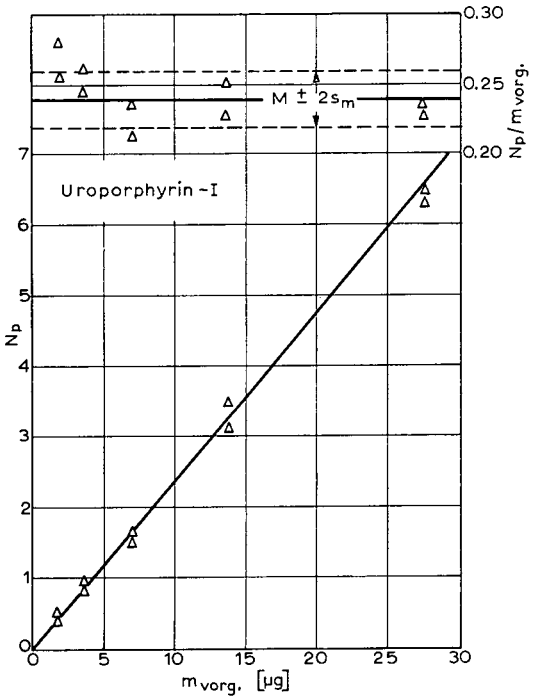


Fig. 14. Quantitative Bestimmung von Uroporphyrin-I im Bereich von 1.73–27.6 μg neben einer konstanten Menge Uroporphyrin-III ($m_{vorg.} = 28.7$ μg) bei einem Peaklagenabstand von $\Delta x_p/\overline{T}_{50} \approx 2$.

übereinstimmen, zeigen, dass bei dieser Bestimmung in dem untersuchten Bereich ($m_{vorg.} = 1.73$–27.6 μg) mit einer Fehlerschwankung von *ca.* $\pm$ 10 % (95 %-Fehler) gerechnet werden muss. Dies ist eine zur Sicherung klinischer Befunde völlig ausreichende Genauigkeit.

Bei der Auftrennung und Bestimmung der Porphyrine in der beschriebenen Weise sind Verluste in merklichem Umfange nicht zu befürchten. So lag der unter extremen Versuchsbedingungen erhaltene Rückgewinn R ($R = m_{vorg.}/m_{gew.}$) für Coproporphyrin und Uroporphyrin bei $R = (99 \pm 5)$ %[13].

ZUSAMMENFASSUNG

Zur Analyse komplexer Porphyringemische, insbesondere zur Bestimmung der Uroporphyrinisomere I und III, wird ein extraktionschromatographisches Verfahren beschrieben, das Trennung und quantitative Bestimmung der in freier Form vorliegenden Hämpräkursoren in einem Arbeitsgang durchzuführen gestattet. Die Auftrennung wird im Verteilungssystem Tri-*n*-butylphosphat/1 *N* Salzsäure auf Säulen hoher Bodenzahl ($N = 300$–450) vorgenommen. Unter den angegebenen Versuchsbedingungen (Säulentemperatur: $T_s = 15$–$30°$: Säulenlänge: $L_s = 120$ cm; Säurekonzentration: $c_{HCl} = 1\ N$) können die beiden Isomere in einem Zeitraum von 20–30 h nahezu völlig aufgetrennt werden.

Zur quantitativen Bestimmung von Uroporphyrin-I, III wird der Verlauf der simultan registrierten Transmission der eine Durchflussküvette passierenden Elutionslösung ausgewertet. Nach Digitalisierung der aufgezeichneten Elutionspeaks und Umrechnung in die entsprechenden Extinktionswerte wird aus der Fläche der einzelnen Peaks, nach vorangegangener Eichung der Versuchsanordnung mit reinen Bezugssubstanzen, die in der Probe vorliegende Porphyrinmenge erhalten.

Die zur vollständigen Auftrennung erforderlichen, beträchtlichen Elutionszeiten können, durch Variation der Versuchsbedingungen, erheblich reduziert werden, falls die Zerlegung der unter diesen Bedingungen komplexen Elutionspeaks mit dem beschriebenen Auswerteverfahren vorgenommen wird. Hierbei können Peaks bei einem Peaklagenabstand von $\Delta x_p/\overline{T}_{50} = 1.5$–$2.0$ (je nach Mengenverhältnis) noch mit ausreichender Genauigkeit ausgewertet werden. Eine Bestimmung der Uroporphyrinisomere I und III ist bei einem Mengenverhältnis von $0.06 \leqq$ UP-I/UP-III $\leqq 16$ bis *ca.* 1 μg mit einer Genauigkeit von *ca.* 10 % (95 %-Fehler) möglich. Verluste an Porphyrinen sind bei Auftrennung und Bestimmung selbst unter extremen Versuchsbedingungen in merklichem Umfange nicht zu befürchten.

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CHROM. 3704

SELECTIVITY PROPERTIES OF POLY-N-VINYL PYRROLIDONE IN COLUMN CHROMATOGRAPHY OF NUCLEOTIDES, THEIR DERIVATIVES, AND RELATED COMPOUNDS: A PRELIMINARY REPORT*

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SUMMARY

Insoluble poly-N-vinyl pyrrolidone (PVP) has been found to possess selectivity properties toward various nucleotide derivatives and can be used for column chromatography employing water as eluent. Compounds emerge from the columns in this order: nucleotides, pyrimidines, purines. Total elution volumes and times for the series of compounds investigated are 45 ml (5 h) with use of a 0.9 × 22.3 cm column and 20 ml (65 min) with use of a 0.9 × 10.8 cm column.

INTRODUCTION

Poly-N-vinyl pyrrolidone can be used effectively as a complexing agent in removing polyphenols from plant enzyme preparations[1–3]. Recently it has been applied in its insoluble form by QUARMBY[1] for effecting thin-layer chromatographic separations of phenolic acids and flavonoids. He found PVP to bind phenolic compounds tenaciously and very polar solvents were necessary to move them from the plate origin. GUSTAVSON[4,5] discovered that PVP-vegetable tannin complexes can be disrupted by treatment with 6–8 *M* urea and that hydrogen bonding to hydroxyl groups of tannins is the main interaction in complex formation. Recently, ANDERSEN AND SOWERS[3] investigated conditions for PVP–plant phenol complex formation and noted binding to increase in the series, scopoletin, caffeic acid, and quercetin, *i.e.*, in the order of increasing number of free hydroxyl groups. They also reported rutin to bond least when its phenolic hydroxyls are dissociated, *e.g.*, in alkaline solution. Likewise the glycoside of scopoletin, scopolin, does not bind the polymer[3]. PVP has also been incorporated into Gas-Chrom P** (a support for use in gas chromatography) where it has been found to increase column affinity toward alcohols and selectivity for

* The following abbreviations will be used: AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; UMP = uridine monophosphate; XMP = xanthosine monophosphate.

** Mention of trade or company names does not imply endorsement by the Department over others not named.

closely related plant sterols[6]. Thus the retention times of dihydroxy alkaloids, such as morphine and reticuline, on a treated support are more than doubled[6]. PVP was also noted to be selective for aromatic moieties as evidenced by the increased retention times of papaverine and cinchona alkaloids, which contain the quinoline function[6]. Another application has been the separation of lipoproteins by flotation in solutions containing sodium chloride and PVP[7].

It is the purpose of this study to offer a preliminary report on the applicability of PVP in separations of certain nucleotide derivatives and related compounds.

METHODS

A commercial preparation of PVP, obtained from the General Aniline and Film Corporation (New York), was used in these studies. The polymer is sold under the trade name of Polyclar AT Powder, which, according to the manufacturer, is a high molecular weight, cross-linked form of PVP and is insoluble in water, organic solvents, acid, and alkali[8,9]. Polyclar AT particles range in size from greater than 60 B. S. mesh (250 μ) to less than 300 B. S. mesh (53 μ) and are known to swell slightly in water[1]. In preparing chromatographic columns, the powder was mixed with distilled water and allowed to settle with repeated decantation of the fines. The slurry was poured into two small columns of identical dimensions, designated A and B (0.9 $\times$ 10.8 cm), and into a longer column C (0.9 $\times$ 22.3 cm). In all experiments, distilled water (pH 6) was used as eluent, and this was delivered to the columns under conditions of room temperature (25°) and atmospheric pressure. Flow rates averaged 0.3 ml/min for columns A and B, and 0.15 ml/min for column C. All compounds were obtained from Nutritional Biochemical Co., except ADP and ATP, which were purchased from Sigma Chemical Co., and were used without further purification. They were applied singly or in mixtures to the columns, in amounts between 0.1 mg and 0.5 mg, and in 0.2 ml distilled water. Eluate was collected in 0.3 or 0.4 ml fractions and monitored at 260 mμ in a Gilford 220 spectrophotometer.

RESULTS AND DISCUSSION

In our initial investigations with PVP, short columns were made to study the selectivity properties of this polymer toward various nucleotides, their derivatives, and related compounds. Table I gives a list of these substances with their respective elution volumes. Band widths are given to illustrate peak spreading. Vitamin B_{12} appeared first in the elution series; this may be due to exclusion from the PVP matrix because of its large size. If this is the case, the amount of its hydrogen and hydrophobic bonding to PVP would be small, because such interactions would be limited to the particle surface. The early elutions of AMP, UMP, and 2'-AMP, despite their possession of bonding groups, may be indicative of a repulsive, electrostatic interaction between the negatively charged phosphate group in the nucleotides and the negatively polarized PVP carbonyl group. No adequate explanation can be given for the elution of riboflavin phosphate at an elution volume greater than that of the nucleotides or riboflavin. In Table I it is seen that the retention volumes of the pyrimidines range from about 9 ml to 10.5 ml. These compounds, as will be discussed below, bond weakly to the PVP matrix. On the other hand, the purines (and adenosine) bond

TABLE I

ELUTION VOLUMES OF NUCLEOTIDES, THEIR DERIVATIVES, AND RELATED COMPOUNDS ON A 0.9 × 10.8 cm COLUMN

Compound	*Elution volume*[a,b] *(ml)*	*Band width*[c] *(ml)*
Vitamin B_{12}	6.0	
AMP	7.4	0.9
UMP	7.9	0.9
2′-AMP	8.0	
Riboflavin	8.1	
5-Aminouracil	8.7, 9.3	0.9
Thymine	9.3, 9.3, 9.7	
Cytosine	9.9	1.0
6-Methyluracil	10.4[d]	
5-Nitrouracil	10.5, 10.5	
Riboflavin phosphate	10.7[d]	
Xanthine	12.6, 12.6	1.6
Adenosine	13.8	1.3
Adenine	18.9, 19.5	1.9

[a] Volume of eluate to maximum concentration.
[b] Each figure is the value obtained in a single experiment.
[c] Peak width at half-maximum concentration.
[d] Indicates elution on column B; other values are from column A.

more strongly to the matrix and therefore are removed last from the column. Thus it is observed that short columns of PVP can be used with water as eluent to effect class separations of certain nucleotides from pyrimidines as well as pyrimidines from purines (and adenosine) in a small volume of eluate. This is done rapidly (in 65 min) under conditions of room temperature and atmospheric pressure.

Selectivity properties of PVP were examined further using a longer column, C, and distilled water again as eluent. Fig. 1 indicates that no separation of the mono-, di-, and triphosphates of adenosine from each other could be effected. Similarly, XMP was eluted with a retention volume of 14.1 ml. This was judged not to be significantly different from the volume obtained for AMP, ADP, and ATP. These nucleotides precede several pyrimidines that are also not separable. Regarding the elutions of two pyrimidines, thymine and uracil, it is assumed that each can form two hydrogen bonds with the pyrrolidone carbonyl groups of PVP. In the keto form, this interaction could involve a proton on the secondary nitrogen atoms. It is also of interest that the methyl group in thymine does not appear to influence retention of this compound. The elution behavior of 5-aminouracil seems to suggest that only two

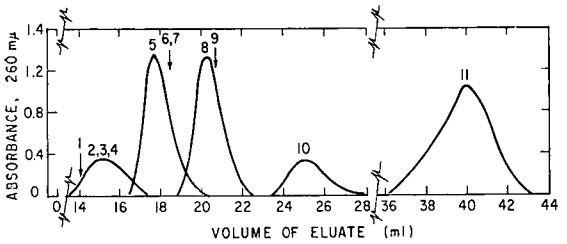

Fig. 1. Chromatogram of nucleotides and bases on column C (0.9 × 22.3 cm). 1 = XMP; 2 = AMP; 3 = ADP; 4 = ATP; 5 = uracil; 6 = thymine; 7 = 5-aminouracil; 8 = hypoxanthine; 9 = xanthosine; 10 = xanthine; 11 = adenine.

hydrogen bonds are formed between this substance and PVP. Thus, it can be postulated that the primary amino group in 5-aminouracil participates in an internal hydrogen bond with its carbonyl thereby forming a five-membered ring.

Although hypoxanthine, like the pyrimidines mentioned, only has two protons available for hydrogen bonding to the column, it has a greater retention volume. Perhaps the reason for this is its larger ring system which provides for a greater degree of nonpolar interactions with the pyrrolidone matrix. COHN[10] has observed that purine bases such as xanthine and hypoxanthine are retained to a small degree even on cation exchange resins, and this he attributes to nonpolar attractions. The influence of hydrogen bonding on elution volume is seen in the case of xanthine which has three available protons and hence a greater retention than hypoxanthine. Also, the strong retention of adenine on column C as well as on Sephadex G-10, as reported by SWEETMAN AND NYHAN[11], can be partly explained by its ability to form hydrophobic bonds to column backbones. Furthermore, while only two protons in this compound can hydrogen bond to PVP, it is thought that the presence of sp^3 orbitals in the primary amino group increases the hydrogen-donating effect by enhancing proton availability as compared with the hydrogen-donating properties of the shorter sp^2 bonds of secondary nitrogen groups. Since the primary amino group of adenine has a pK of 4.1[10], hydrogen bonding at pH 6 involves about 1 % of the charged and about 99 % of the uncharged species.

The elution of xanthosine in a retention volume of 20.7 ml (Fig. 1) indicates the effect of the ribofuranosyl moiety in reducing elution volume as compared to the free base (see data for adenine and adenosine in Table I).

Table II gives reproducibility and recovery characteristics of a PVP column. Application of a mixture of three bases to column C was repeated three times on different days. The data show that the column can be used repeatedly with only small variability in retention volume for a given compound. Similarly recovery values were found to be high. The lack of tailing (Fig. 1) is also indicative of reversible adsorption of solute. The elution profiles obtained on this column are generally symmetrical,

TABLE II

REPRODUCIBILITY AND RECOVERY CHARACTERISTICS OF CHROMATOGRAPHY ON PVP

Reproducibility experiment: application of a mixture of uracil, xanthine, and adenine to column C; eluent, distilled water. Recovery experiment: application of xanthosine, ATP, and hypoxanthine singly to column C; eluent, distilled water.

Compound	*Retention volume (ml)*		
	Run 1	*Run 2*	*Run 3*
Uracil	17.6	17.6	17.7
Xanthine	25.4	25.1	24.9
Adenine	41.3	41.9	40.0
	Amount applied (mg)		*% recovered*
Xanthosine	0.13		93
ATP	0.13, 0.50		100, 100
Hypoxanthine	0.29		88

except for adenine, which tends to show some peak "heading", and the separation of classes is good.

Some advantages are evident in the use of water; it is a mild eluent and allows sample recovery uncontaminated with salts. In reference to solute sorption effects, hydrogen bonding in a distilled water system undoubtedly is more effective than in eluents containing electrolytes. In the latter case, salt can disrupt polymer–solute interactions.

The use of small columns, although limiting sample size, permits collection in a minimal volume of eluate. With the longer column (C) it is possible to separate certain purines, nucleotides from pyrimidines, and pyrimidines from purines (and xanthosine) in a volume of water less than 45 ml (in about 5 h). This system should have applicability for rapid preliminary separation of these classes of compounds.

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CHROM. 3665

THE SEPARATION OF RIBONUCLEIC ACIDS ON SEPHADEX COLUMNS

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SUMMARY

A method was developed for the chromatographic separation of soluble ribonucleic acid (sRNA) and ribosomal ribonucleic acid (rRNA). It involves the use of Sephadex G-75, which was equilibrated with 1.5 *M* NaCl and 0.5 *M* Tris buffer, pH 7.5. After the mixture was embedded, the column was eluted with 0.5 *M* Tris buffer using a 50 ml mixing pot. Excellent separation of sRNA and rRNA was obtained from purified samples as well as crude samples isolated from a human leukemic lymphoblast. Column recoveries were essentially 100%, and the method is fast and very reproducible. Other advantages, such as column capacities, non-denaturation and completeness of separation are discussed.

There are two general procedures usually employed for the separation of soluble ribonucleic acid (sRNA) from ribosomal ribonucleic acid (rRNA). One involves the isolation of the ribosomes from the cytoplasm by ultracentrifugation followed by the precipitation of the respective RNAs, as has been described with yeast[1], *E. coli*[2], and liver[3,4]. The other methods involve the fractionation of whole cell RNA, usually by selective precipitation with NaCl[5], LiCl[6], or streptomycin[7], followed by further purification such as column chromatography[8–10].

During the course of studies involving human leukemic lymphoblasts (CCRF-CEM), a method was developed for the separation of sRNA from rRNA which is applicable to whole cell crude RNA fractions. The procedure is based on "salting out" rRNA on a Sephadex G-75 column, using a solvent of 1.5 *M* NaCl, 0.05 *M* Tris pH 7.5. The sRNA passes through the column in the first few fractions and rRNA emerges in later fractions during a gradient elution with 0.05 *M* Tris pH 7.5. The method is rapid and easily reproducible. No expensive equipment is required, no denaturation occurs and the recoveries are essentially complete. The present report describes this procedure.

MATERIALS AND METHODS

Sephadex G-75-120 was equilibrated (72 h) with 100 ml of a solvent system containing various concentrations of NaCl and 0.05 *M* Tris pH 7.5, and fines were

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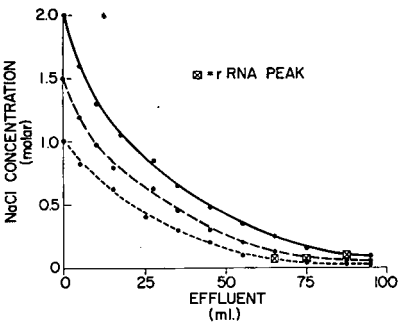
removed three times by aspiration. While the original studies were conducted with 9 mm × 25 cm columns, subsequent investigations utilized 15 mm × 25 cm columns (Lab Glass, Inc., Vineland, N.J.). One and 2.3 g of Sephadex were used in the 9 and 15 mm columns respectively. Before the Sephadex was introduced into the column, 2 ml of a 10% suspension of Whatman cellulose CC-31 (w/v) was placed directly upon the fritted glass disc. The gel was then introduced into the column and flushed with ten volumes of solvent. One milliliter aliquots of the samples were introduced on the column, followed by two 1 ml portions of solvent to embed the material. The column was then filled with NaCl–Tris solution, a 50 ml mixing pot was attached to the column and connected to a reservoir containing 0.05 *M* Tris, pH 7.5 for elution.

The batch-production of CCRF-CEM cells (20–25 g wet weight per 15 l suspension culture) has been described elsewhere[11]. Cells were harvested from such suspension cultures in a continuous flow Sorvall centrifuge, and whole cell crude RNA was extracted after removal of DNA according to the method described by KAY[12]. For control studies, sRNA and rRNA were isolated from yeast according to the methods described by HOLLEY *et al.*[13] and CRESTFIELD *et al.*[14], respectively. RNA was determined by ALBAUM AND UMBREIT'S modification of the orcinol method[15], and sodium determinations (calculated as NaCl) were made in a Perkin Elmer flame photometer.

RESULTS

Yeast rRNA was embedded on the several columns which had been equilibrated with different concentrations of NaCl. The columns were then eluted (by gradient) with Tris buffer, and some results are illustrated in Fig. 1. The rRNA peaks emerged at the 65, 75, and 85 ml fractions when the initial solvent system contained 1.0, 1.5, and 2.0 *M* NaCl, respectively, and the concentrations of NaCl in the eluates containing rRNA was found to be of the order of 0.01 *M*. When sRNA was embedded on the



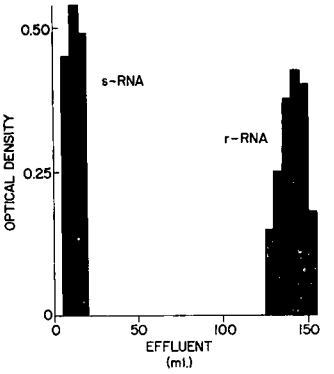


Fig. 1. The gradient elution of rRNA when embedded with solutions containing different concentrations of NaCl in Tris buffer. The mixing pot was 50 ml of embedding solution, and a solution of 0.05 *M* Tris buffer, pH 7.5 was the eluting solvent. For experimental details, see text.

Fig. 2. A typical elution pattern for the separation of sRNA from rRNA. The column was charged with a mixture of yeast sRNA (2.0 mg) and rRNA (2.0 mg). The column used was 15 mm × 25 cm containing 2.3 g of Sephadex G-75-120 equilibrated with 1.5 *M* NaCl, 0.05 *M* Tris, pH 7.5, and eluted with 0.05 *M* Tris buffer. A 50 ml mixing pot was used for the gradient elution.

column, the peaks were consistently eluted in the first 15 ml irrespective of the concentration of NaCl used. As a result, 1.5 *M* NaCl and 0.05 *M* Tris, pH 7.5 was selected as the embedding solution.

When mixtures of yeast sRNA and rRNA were placed on a 15 mm column, the peaks emerged in slightly different fractions (as compared to the 9 mm column). A typical elution pattern is shown in Fig. 2. sRNA was found between the 5th and the 20th ml while rRNA emerged in the 125th to 155th ml fractions. The capacity of the column was then tested and satisfactory separations were obtained when as much as 20 mg each of sRNA and rRNA were used. U.V. analyses were made in a Beckman DU spectrophotometer and sedimentation coefficients were determined on the starting material and the eluted samples. Both the initial and final material had the same absorbance and the same S rates.

Some recovery experiments were done to determine the column efficiency. Table I shows the results, using purified yeast sRNA and rRNA. With the exception of the 0.16 mg sample of rRNA, the recoveries from chromatography are in the order of 95–100 %, irrespective of whether the samples were embedded individually or

TABLE I

RECOVERY OF ISOLATED YEAST sRNA AND rRNA FROM SEPHADEX G-75 COLUMNS

Amount added (mg)		*Amount recovered (mg)*		*% recovered*	
sRNA	*rRNA*[a]	*sRNA*	*rRNA*	*sRNA*	*rRNA*
0	0.16	0	0.14	—	88
0	0.40	0	0.39	—	97
2.0	0	1.99	0	99	—
5.0	0	4.98	0	99	—
5.0	0.40	4.80	0.39	96	97

[a] Small amounts of rRNA were used due to the limited amount of material available.

TABLE II

RECOVERY OF YEAST sRNA AND rRNA AND HUMAN LEUKEMIC LYMPHOBLAST RNA FROM SEPHADEX G-75 COLUMNS

Milligrams added			*Total milligrams found*		*Yeast RNA*			
					Milligrams recovered[a] *(calc.)*		*% recovered*	
Crude CCRF-CEM RNA fraction[b]	*Yeast RNA*[c] *sRNA*	*rRNA*	*sRNA*	*rRNA*	*sRNA*	*rRNA*	*sRNA*	*rRNA*
7.2	—	—	0.94	2.25	—	—	—	—
7.2	28.0	—	29.16	—	28.22	—	101	—
14.4	—	10.0	1.71	14.20	—	9.70	—	97
7.2	30.0	7.0	30.03	9.20	29.09	6.95	97	99

[a] Milligrams of yeast RNA recovered is calculated by subtracting the RNA found in the crude CCRF-CEM fraction from the total milligrams found. This calculated value is then compared to the amount of purified yeast RNA added to determine % recovery.

[b] The crude fraction was isolated by the method of KAY after removal of DNA[12].

[c] Commercial source: RNA (soluble) Type III and rRNA type XI (Sigma, St. Louis, Mo.).

as a mixture. When purified yeast sRNA was added to CCRF-CEM whole cell crude RNA, the recoveries of yeast RNA were approximately 100 % (see Table II). It was of interest to note that the whole cell crude RNA fraction contained approximately 43 % RNA. This, however, should not be considered as a reflection on the method of isolation, since KAY's procedure was not designed to isolate RNA in purified form[12]. Nevertheless, the column recoveries support the validity of the presently reported separation method.

DISCUSSION

There have been a number of chromatographic methods reported for the separation of RNAs. For example, DEAE cellulose[16,17], and ECTEOLA cellulose[18,19], have been used to develop satisfactory separation procedures. However, some degradation of rRNA occurs when these materials were used. Methylated serum albumin on Kieselguhr has been similarly used[20], but this system has a very low column capacity. Recently, BARBER[21] reported the use of unmodified cellulose and NaCl–ethanol solvent system for the separation of these nucleic acids. sRNA was recovered in the 2nd to the 13th (5 ml) fraction. At the end of the 14th fraction, the eluting solvent was changed to distilled water and rRNA was collected in the 15th to the 20th fraction. When ^{14}C-isoleucine-labeled sRNA was used on the unmodified cellulose column, the resulting elution pattern suggested a slight overlapping of the sRNA and rRNA fractions. With respect to column capacity, BARBER's method is superior to columns containing methylated albumin on kieselguhr in that 15 mg of rRNA could be adsorbed and recovered from a column containing 4 g of Whatman's cellulose powder CF-11. In the present studies, 2.3 g of Sephadex G-75 could readily separate 20 mg each of sRNA and rRNA. U.V. absorption and S-rates of the purified yeast RNAs before and after chromatography were found to be the same. This, in conjunction with the recovery experiments, indicated that no degradation of RNA occurred while it was on the column.

Some mention should be made of the relative amounts of sRNA and rRNA isolated from the CCRF-CEM cells. While KAY's method[12] of isolation was not intended to be quantitative, rRNA represented more than 70 % of the total RNA isolated from the cell. This observation is of particular interest since MCCARTHY *et al.*[22] reported that cytochemical analyses of the CCRF-CEM cells indicated a mean RNA:DNA ratio of 0.7:10, which is somewhat unusual for mammalian cells. These observations will be considered in more detail elsewhere.

The method of separating sRNA from rRNA described herein is easy to run, and is highly reproducible. It is readily applicable to the use of fraction collectors and does not require any attention during elution. The recoveries are approximately 100 %, no denaturation occurs, the column capacities are high, and it is readily applicable to whole cell RNA isolated from mammalian cells.

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CHROM. 3653

ÉTUDE DES FRACTIONS OBTENUES PAR CHROMATOGRAPHIE DU VENIN DE *NAJA NAJA ATRA* SUR SULPHOÉTHYL-SEPHADEX

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SUMMARY

A study of the fractions of the venom of Naja naja atra obtained by chromatography on sulphoethyl-Sephadex

The venom of *Naja naja atra* was fractionated on sulphoethyl-Sephadex into fourteen clearly different fractions. The experimental conditions are described. A number of enzymatic activities were studied, *viz.* phospholipase A, cholinesterase, L-amino acid oxidase, adenosine triphosphatase, 5'-nucleotidase, ribonuclease, phosphodiesterase, nicotinamide dinucleotide phosphatase and hyaluronidase, as well as several inhibiting activities of enzymatic processes, *viz.* inhibition of anaerobic glycolysis, inhibition of the cytochrome oxidase system and inhibition of acetylcholinesterase. The following biological activities are described: toxicity, anticoagulation, cytotoxicity, and the direct haemolytic factor. The well separated fractions were analysed by electrophoresis on acrylamide gel and their molecular size was estimated by means of gel filtration.

INTRODUCTION

Depuis quelques années, les venins de serpents sont l'objet d'un intérêt toujours plus vif, non seulement parmi les chercheurs engagés dans l'étude des toxines, mais aussi et surtout parmi les biochimistes, qui y trouvent un éventail enzymatique incomparable, et parmi les pharmacologistes, qui voient, dans les nombreuses activités physiologiques des venins, des ressources thérapeutiques nouvelles.

L'étude de ces activités exige l'isolement préalable, à l'état pur, des composés qui en sont responsables.

Les dextranes modifiés, commercialisés en 1962, ont depuis lors, grâce à leur excellent pouvoir de résolution, fait largement leurs preuves dans la séparation des constituants de complexes protéiques. Cependant cette méthode n'a, jusqu'à présent, été que très peu appliquée au fractionnement des venins de cobra. A notre connaissance les seules expériences effectuées dans ce domaine sont celles de VICK, CIUCHTA, BROOMFIELD ET CURRIE[32] qui, en 1966, utilisèrent le fractionnement sur carboxyméthyl-Sephadex pour l'isolement de fractions physiologiquement actives d'un venin

de cobra et celles de LO, CHEN ET LEE[21] qui, en 1967, utilisèrent le même support pour le fractionnement primaire d'un venin de *Naja naja atra*.

La plupart des constituants protéiques du venin de *Naja naja atra* étant de nature basique, l'utilisation pour leurs fractionnements de gels échangeurs cationiques du type carboxyméthyl ou sulphoéthyl semblait s'imposer. Les approches expérimentales nous ont conduits[30] à choisir le sulphoéthyl-Sephadex qui semblait donner une résolution nettement supérieure.

L'objet du présent article est de décrire les conditions expérimentales de ce mode de fractionnement, de fournir, pour les différentes fractions obtenues, les rendements pondéraux, les caractéristiques spectrales, les activités enzymatiques et inhibitrices d'enzymes ainsi que la localisation de certaines activités biologiques.

Pour les fractions présentant une bonne résolution, les analyses électrophorétiques en gel d'acrylamide et les profils de filtration sur une colonne calibrée en poids moléculaire seront également étudiés.

MATÉRIEL

Matériel biologique

Venin. Nous avons utilisé comme matériel de départ un échantillon de venin de cobra (*Naja naja atra*) qui nous a été aimablement fourni sous forme lyophilisée par le professeur C. C. YANG du Kaohsiung Medical College de Formose.

Le venin est conservé à +4°, à l'abri de l'humidité et de la lumière.

La toxicité, dose léthale à 50 %, de l'échantillon utilisé lors d'injections par voie intrapéritonéale est de 400 μg/kg. Il contient 0.54 % de Zn et 0.25 % de Mg. L'analyse électrophorétique en gel de polyacrylamide fait apparaître 17 disques[12]. La filtration sur gel (Sephadex G 50) fait apparaître 6 pics et montre la présence d'une forte proportion de substances à faible poids moléculaire (cf. Fig. 3).

Cellules. Les cellules ascitiques d'Ehrlich sont originaires de l'Institut Mario Negri de Milan (Prof. GARATTINI). Elles sont maintenues sur des souris par inoculations péritonéales hebdomadaires de 0.2 ml d'une dilution à 20 % dans du sérum physiologique du fluide ascitique collecté entre le 7ème et le 10ème jour après l'inoculation.

La densité cellulaire est évaluée par comptage à la cellule de Thomas ou au Coulter counter et est ajustée à la concentration voulue par dilution au moyen d'une solution isotonique.

Matériel chimique

L'eau utilisée est désionisée; tous les réactifs sont de qualité analytique.

Les membranes d'ultrafiltration proviennent de Membranfilter, Göttingen. Les gels Sephadex et les Sephadex modifiés sont fournis par Pharmacia, Uppsala, tandis que le Bio-Gel est obtenu chez Bio-Rad, Richmond, Calif.

MÉTHODES

Dosage des protéines

Une unité optique à cellule de passage (Uvicord II, LKB) permet de mesurer en continu la D.O. à 280 mμ de l'éluat à la sortie de la colonne de chromatographie.

Les protéines sont dosées par mesure des densités optiques à 260 et 280 mμ relevées en cellules de 1 cm grâce à un spectrophotomètre Unicam SP 800 ainsi que par la méthode colorimétrique de LOWRY[22] dérivée de la méthode de FOLIN[4].

Essais enzymatiques

La *phospholipase A* (phosphatide acyl-hydrolase, E.C. 3.1.1.4.) est déterminée suivant la méthode de NEUMANNN ET HABERMANN[23].

La *cholinestérase* (acylcholine acyl-hydrolase, E.C. 3.1.1.8.) est mesurée suivant la méthode colorimétrique de KRAMER ET GAMSON[18].

La *L-amino-acide oxydase* (L-amino-acide O_2 oxydoréductase, E.C. 1.4.3.2.) est estimée manométriquement suivant ZELLER ET MARITZ[36].

L'adénosine triphosphatase (adénosine triphosphate-pyrophosphohydrolase, E.C. 3.6.1.8.) est mesurée suivant YANG *et al.*[34].

Les activités *ribonucléasique* (polyribonucléotide 2-oligonucléotidotransférase, E.C. 2.7.7.16.) et *déoxyribonucléasique* (déoxyribonucléate oligonucléotidohydrolase, E.C. 3.1.4.5.) sont testées suivant la méthode de SHAPIRA[29].

La *5'-nucléotidase* (5'-ribonucléotide phosphohydrolase, E.C. 3.1.3.5.) est estimée suivant la méthode de BABKINA ET VASILENKO[2].

La *phosphodiestérase* (orthophosphate diester phosphohydrolase, E.C. 3.1.4.1.) est dosée par la méthode spectrophotométrique de BOMAN ET KALETTA[5].

La *NADase* (nicotinamide dinucléotide phosphohydrolase, E.C. 3.6.1.9.) est mesurée par détermination de la vitesse de clivage du NADH suivant la technique de REEVES ET FIMOGNARI[27].

L'hyaluronidase (hyaluronate lyase, E.C. 4.2.99.1.) est estimée selon la méthode turbidimétrique de KASS ET SEASTONE[17], modifiée par TOLKSDORF[31].

Inhibiteur de la glycolyse anaérobie. Le taux de glycolyse anaérobie des cellules ascitiques d'Ehrlich ($10 \cdot 10^6$/ml) est déterminé à l'appareil de WARBURG par mesure du dégagement de CO_2 formé à partir de bicarbonate lors de l'apparition d'acide lactique dans le milieu[33].

Inhibiteur du système cytochrome oxydase. L'inhibition du système cytochrome oxydase est mesurée selon la méthode de CHATTERJEE[10] basée sur l'oxydation d'un substrat *p*-phénylènediamine.

Inhibiteur de l'acétylcholinestérase. L'inhibition de l'acétylcholinestérase est mesurée selon la méthode manométrique d'AMNON ET VOSS[1] citée par LEE *et al.*[20]. On utilise comme source d'acétylcholinestérase vraie le venin de *Bungarus coeruleus* (acetylcholine acetyl-hydrolase, E.C. 3.1.1.7.).

Méthodes biologiques

Détermination des toxicités. Pour la détermination des toxicités, des souris Swiss de 25–30 g reçoivent des injections intrapéritonéales de doses croissantes du produit à tester en solution dans 0.2 ml de sérum physiologique.

La LD_{50} (Bibl. 3) est la dose (mg/kg) qui tue 50 % des souris en 24 h. La mortalité est toutefois contrôlée pendant au moins 72 h.

Activité anticoagulante. La détermination de l'activité anticoagulante est basée sur une méthode que nous avons mise au point et qui consiste en une mesure turbidimétrique du temps de coagulation du plasma de cheval citraté et recalcifié.

Cytotoxicité. La cytotoxicité est déterminée sur des cellules ascitiques du car-

cinome d'Ehrlich. On mesure le pourcentage de cellules devenues perméables au vert de Lissamine[16] après incubation pendant 3 h dans du sérum physiologique à 37° en présence de différentes doses de substance à tester.

Hémolyse directe. Le facteur hémolytique direct (DLF) est mis en évidence par mesure spectrophotométrique à 540 mμ de l'hémoglobine libérée lors de l'incubation de globules rouges ($200 \cdot 10^6$/ml de sérum physiologique) en présence des produits à tester à 30° pendant 60 min[13].

Méthodes physico-chimiques

Spectres ultraviolets. Les spectres ultraviolets des substances obtenues ont été relevés en cuvettes de 1 cm au moyen d'un spectrophotomètre Unicam SP 800.

Electrophorèse en gel de polyacrylamide. Les électrophorèses en gel de polyacrylamide sont effectuées suivant la méthode d'ORNSTEIN ET DAVIS[25,26] (gel à 7.5 % de monomère; pH 8.9) pour les protéines à caractère acide, et suivant la méthode modifiée de REISFELD, LEWIS ET WILLIAMS[28] (gel à 15 % de monomètre; pH 4.3) pour les protéines basiques[12].

Filtration sur gel — répartition des poids moléculaires. Les fractions sont soumises à la filtration sur gel de dextrane (Sephadex G 50 fine) sur une colonne de 200 cm de haut et de 1 cm de diamètre, en utilisant comme éluant une solution d'acétate d'ammonium 0.1 *M*, pH 6.8.

TABLEAU I

STANDARDS UTILISÉS POUR CALIBRER LA COLONNE DE FILTRATION SUR GEL

Substance	*Abréviations utilisées*	*Origine*	*Poids moléculaire*
Vitamine B_2	B 2	Roche (Bâle)	376
Vitamine B_{12}	B 12	Uclaf (Paris)	1,355
ACTH synthétique	ACTH	Organon	3,500
Cytochrome C	Cyt. C	Sigma	12,400
Ribonucléase A	RNAse	Sigma	13,700
α-Chymotrypsine	α-Chym.	Koch-Light	25,000
Blue-Dextran	BD	Pharmacia	2,000,000

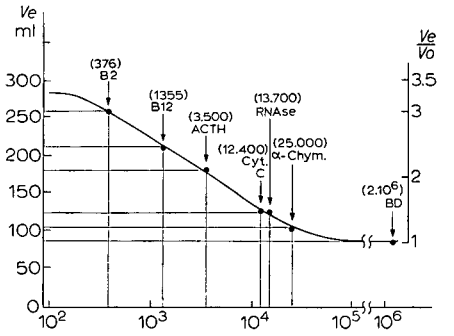

Fig. 1. Relation entre le poids moléculaire et le volume d'élution. Colonne: Sephadex G 50 fine: diamètre 1 cm, hauteur 200 cm. Éluant: acétate d'ammonium 0.1 *M*, pH 6.8. Vitesse d'élution: 0.17 ml/min (pompe péristaltique). Température: 20–25°. Abréviations utilisées, voir le Tableau I.

Les essais sont effectués à la température ambiante. Le débit de la colonne est imposé et maintenu constant par une pompe péristaltique située à la sortie de la colonne; il est de 0.17 ml/min. La pression hydrostatique de l'éluant au-dessus de la colonne est de 20 cm; elle est maintenue constante par une alimentation continue en éluant.

Le calibrage de la colonne est effectué au moyen de différentes substances de poids moléculaires connus (Tableau I, Fig. 1). Dans les conditions opératoires utilisées, les volumes d'élution sont reproductibles à ± 1 %.

RÉSULTATS

Fractionnement

Des approches effectuées sur microcolonnes (diamètre 0.5 cm, hauteur 20 cm) au moyen de différents supports et pour différents modes d'élution nous ont permis[30] de déterminer les conditions optimales de fractionnement du venin de cobra.

C'est sur base de ces essais d'orientation que nous avons soumis le venin de *Naja naja atra* au fractionnement sur sulfoéthyl-Sephadex dans les conditions décrites ci-dessous.

Le SE Sephadex C 25 (40–120 μ), après traitements successifs par NaOH 0.5 *M*, NaCl 0.5 *M* et HCl 0.5 *M* suivant le protocole habituel, est équilibré à pH 6.0 au moyen d'un pH-stat.

Après une dernière filtration sur verre fritté, le gâteau est remis en suspension dans un tampon phosphate 0.05 *M*, pH 6.0. Le gel est introduit dans une colonne pour former un lit homogène de 1.5 cm de diamètre et de 25 cm de haut, et enfin lavé abondamment par le même tampon.

Un échantillon de 500 mg de venin lyophilisé de cobra formosan (*Naja naja atra*) est dissous dans 10 ml du tampon phosphate 0.05 *M*, pH 6.0. L'élution est effectuée au moyen d'un appareil à gradient à deux chambres identiques[15] suivant le protocole détaillé au Tableau II.

La vitesse d'élution est réglée en sortie de colonne par une pompe péristaltique maintenant un débit de 0.17 ml/min.

Des fractions de 6 ml sont collectées automatiquement. La courbe d'élution (Fig. 2) est suivie en continu par mesure de l'extinction à 280 mμ.

TABLEAU II

SCHÉMA D'ÉLUTION

	Chambre	*Étape I*		*Étape II*		*Étape III*		*Étape IV*	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Volume (ml)		200	0	200	200	250	250	250	250
$[PO_4^{3-}]$		0.05 *M*	—	0.05 *M*	0.05 *M*	0.05 *M*	0.05 *M*	0.05 *M*	0.05 *M*
[NaCl]		0	—	0	0.33 *M*	0.30 *M*	0.50 *M*	0.45 *M*	1 *M*
pH		6.0	—	6.0	6.0	6.0	6.0	6.0	6.0

TABLEAU III

RENSEIGNEMENTS GÉNÉRAUX

	No. fractions													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Rendement pondéral	15%	0.5%	2%	1.5%	1%	2.2%	4%	3%	7%	11%	5%	8%	5%	1%
Folin	+	−	+	+	+	+	+	+	+	+	+	+	+	+
D.O. 280 mμ / D.O. 260 mμ	1	0.75	1.5	1.2	1.2	1.3	1.7	1.6	1.5	1.3	1.6	1.1	1.5	1

TABLEAU IV

COMPOSITION ENZYMATIQUE DES FRACTIONS

	No. fractions													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Phosphodiestérase	0	0	0	0	0.42	4.06	3.47	0	0	0	0	0	0	0
5′-Nucléotidase	0	0	0	0.05	0	0	0	0.06	0.45	0	0	0	0	0
ATPase	−	−	−	+	−	−	−	−	+	−	−	−	−	−
RNAse	0	0	0	0	0	0	0	0	6.5	0	0	0	0	0
NADase	0	0	1	1	1	2	1	0	0	0	0	0	0	0
Cholinestérase	3.6	0	0	0.7	0	0	0	0	0	0	0	0	0	0
L-Amino acide oxydase	+++	0	0	++	0	0	0	0	0	0	0	0	0	0
Lécithinase A	++	0	+++	0	0	0	0	0	0	0	0	0	0	0
Hyaluronidase	0	0	0	0	0	0	0	0	0	0	+++	+	0	0

TABLEAU V

ACTIVITÉS INHIBITRICES

	No. fractions													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Inhibition glycolyse anaérobie	o	o	o	o	o	o	o	o	o	o	o	+++	+	o
Inhibition cytochrome C oxydase	+++	o	+	o	o	o	o	o	o	o	o	o	o	o
Inhibition acétyl-cholinestérase	o	o	o	o	o	o	o	o	o	o	+++	o	o	o

TABLEAU VI

ACTIVITÉS BIOLOGIQUES

	No. fractions													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Activité anticoagulante	+++	o	o	o	o	o	o	o	o	o	o	o	o	o
Hémolyse directe	o	o	o	o	o	o	o	o	o	o	o	o	+++	o
Perméabilité vert lissamine	o	o	o	o	o	o	o	o	o	o	o	+++	+	o
Toxicité + de	10,000	10,000	5,000	10,000	2,500	10,000	10,000	2,500	500	5,000	7,500	2,500	4,000	10,000
LD_{50} (μg/ml) — de	—	—	10,000	—	5,000	—	—	3,500	1,000	7,500	10,000	3,500	5,000	—

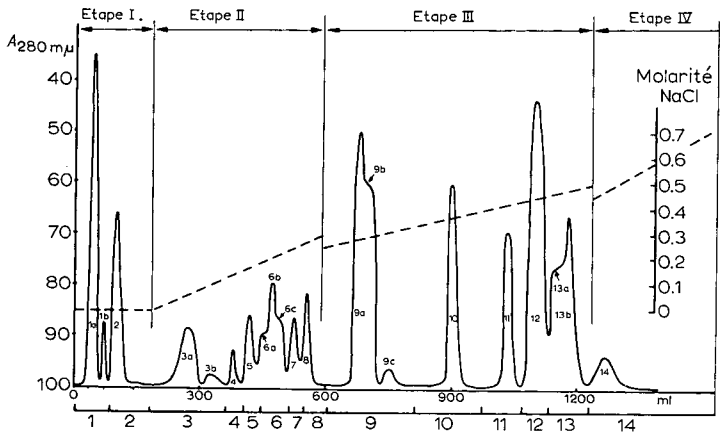

Fig. 2. Fractionnement du venin de *Naja naja atra* sur SE-Sephadex C 25. Colonne: diamètre 1.5 cm. Hauteur: 25 cm. Échantillon: 500 mg venin lyophilisé. Débit: 0.17 ml/min (pompe péristaltique). Tampon: PO_4^{3-}, 0.05 *M*. Gradients linéaires: NaCl 0 → 0.6 *M*.

Dessalination

Les tubes issus du fractionnement sont regroupés sur base du profil d'élution (Fig. 2) et les fractions ainsi obtenues sont dessalinées soit par ultrafiltration au travers d'une membrane de collodion, soit par filtration sur une colonne de gel moléculaire Bio-Gel P_2 (hauteur 90 cm, diamètre 1.5 cm); l'élution se fait par de l'acétate d'ammonium 0.1 *M*, pH 6.8.

Caractéristiques des fractions

Les fractions obtenues sont caractérisées par leur rendement, leur teneur en protéines contrôlée par le réactif de FOLIN ET CIOCALTEU[14] et leurs caractéristiques spectrales (Tableau III), par leurs activités enzymatiques (Tableau IV) et "anti-enzymatiques" (Tableau V), et par un certain nombre d'activités que nous avons regroupées sous le vocable de "biologiques" (Tableau VI).

Les rendements ont été calculés sur base des pesées des fractions après lyophilisation.

Le rendement pondéral obtenu en sommant les rendements partiels est de 68.5 %. Sachant que 7 à 10 % du venin s'échappe à l'ultrafiltration, on peut admettre un rendement global de 75 %. Les différentes fractions présentent des spectres normaux de protéines avec un maximum à 279 mμ et un minimum à 251 mμ. Seules les fractions No. 2 et No. 14 présentent des spectres particuliers.

Signalons enfin que la fraction No. 1 présente une coloration jaune qui peut être attribuée à la riboflavine.

FILTRATION SUR GEL — ÉLECTROPHORÈSE

A titre de contrôle et en vue de déterminer la répartition des masses moléculaires dans les fractions obtenues, un certain nombre de celles-ci ont été soumises à la filtration sur gel Sephadex G 50 sur une colonne analytique préalablement calibrée

au moyen de substances de référence de poids moléculaire connu (Tableau I). La courbe de calibrage de cette colonne est donnée à la Fig. 1.

Les courbes d'élution obtenues pour les substances de référence, pour le venin total (*Naja naja atra*) et pour certaines fractions sont données à la Fig. 3. (Nous n'avons pas jugé nécessaire de reproduire les profils d'élution des fractions 3 à 7 du fait du peu de résolution dans cette zone.) Pour chacune de ces fractions, les images électrophorétiques (gel d'acrylamide) placées vis-à-vis des profils d'élution fournissent une indication de la complexité de la fraction considérée. Dans ces électrophérogrammes schématiques, la flèche symbolise le point d'application, la partie droite

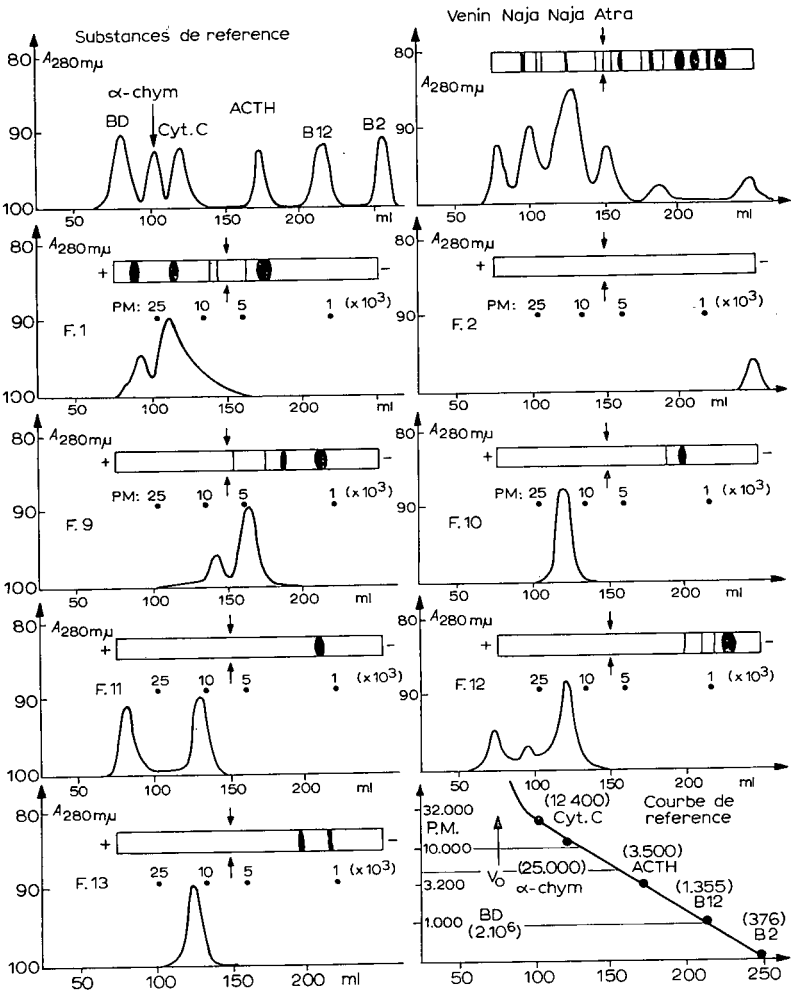

Fig. 3. Étude comparative par électrophorèse en disque et par filtration sur gel Sephadex G 50 des fractions obtenues lors de la chromatographie sur SE-Sephadex du venin de *Naja naja atra*. Les fractions peuvent être comparées au venin total et à un certain nombre de substances de référence utilisées pour calibrer la colonne de dextrane.

représente l'électrophorèse en milieu acide (pH 4.3) et la partie gauche l'électrophorèse en milieu alcalin (pH 8.9).

CONCLUSIONS ET DISCUSSION

L'étude enzymatique ou pharmacologique approfondie d'un constituant d'un venin nécessite d'obtenir ce constituant à l'état pur. Les techniques classiques de purification (les précipitations, par exemple) comportent une suite de processus à faible pouvoir de résolution et conduisent à un simple enrichissement en une espèce moléculaire au détriment de toutes les autres.

Ces méthodes sont en voie de disparition: elles sont progressivement remplacées par des techniques nouvelles, fondées principalement sur la chromatographie et l'électrophorèse préparative. On peut dès lors entrevoir la possibilité du fractionnement intégral d'un complexe tel que le venin de cobra et l'obtention simultanée d'un grand nombre de ses composants à l'état hautement purifié.

Les résultats présentés dans cette étude veulent être une contribution à cette évolution des techniques. En effet, par l'association d'un fractionnement à haut pouvoir résolutif comme le SE Sephadex et de la filtration sur gel moléculaire, on peut espérer obtenir un certain nombre de fractions à l'état fortement purifié.

Le procédé décrit dans cet article permet d'obtenir quatorze fractions. Par un examen attentif de la courbe d'élution, on en dénombre même une vingtaine.

Les spectres ultra-violets et les analyses des fractions au réactif de FOLIN font nettement apparaître la nature non protéique (principalement nucléotidique) du pic 2 et celle non exclusivement protéique du pic 14.

Les différentes activités enzymatiques apparaissent relativement bien séparées et leurs coefficients de purification sont dans la plupart des cas satisfaisants.

La fraction No. 1 contient des substances acides à pH 6.0; nous avons pu notamment y localiser la cholinestérase, l'activité anticoagulante et la lécithinase A qui, comme on sait, inhibe le système enzymatique de la respiration mitochondriale par destruction d'un facteur lipidique important entre le cytochrome B et le cytochrome C[24].

Le FAD passe également dans ce premier pic (1a). La séparation des constituants contenus dans cette première fraction pourrait probablement se faire assez aisément par passage sur une colonne d'échangeur anionique.

La fraction No. 2 est essentiellement nucléotidique.

Le groupe de fractions portant les No. 3 à 8 représente la zone de résolution la moins bonne. Cette zone contient presque tous les enzymes.

La fraction No. 9 s'est avérée la plus toxique.

Cette toxicité semble pouvoir être attribuée à la cobrotoxine[35]. Du point de vue enzymatique, elle contient la RNAse enrichie 6.5 fois, ainsi que la 5'-nucléotidase et l'ATPase pour lesquelles le rendement enzymatique est nettement moins bon.

Il est cependant bien connu que ces deux dernières enzymes sont particulièrement fragiles et il se pourrait que certains moyens préventifs de protection permettent d'éviter cette chute d'activité[4].

La fraction No. 10, bien que se présentant sous une forme relativement purifiée, n'a pu jusqu'à présent être identifiée à un composant connu du venin. Aucune activité enzymatique ni biologique n'a pu y être décelée.

L'inhibiteur de l'acétylcholinestérase a pu être localisé dans la fraction No. 11; les profils d'élution obtenus pour cette fraction lors de la filtration sur gel Sephadex G 50 nous laissent espérer la possibilité d'une bonne purification de cet inhibiteur par couplage des deux techniques.

La fraction No. 12 agit comme inhibiteur de la glycolyse anaérobie[9] et contient le facteur capable de provoquer la perméabilité de cellules au vert de lissamine. Ces deux facteurs pourraient n'être que deux manifestations d'un même phénomène et correspondre au facteur P6 cytotoxique vis-à-vis des cellules du sarcome de Yoshida décrit par BRAGANCA *et al.*[6,7]. Il est par ailleurs très probable, au vu de ses propriétés, que la protéine B 12 décrite tout récemment par LARSEN ET WOLFF[19] comme ayant des activités inhibitrices vis-à-vis des phénomènes de transport au niveau de la membrane de cellules adipiques soit une troisième manifestation de la même substance.

La substance hémolytique directe (DLF)[11] semble pouvoir être complètement dissociée des activités précédentes et a pu être localisée dans la fraction No. 13.

L'utilisation analytique de la filtration sur gel nous a permis non seulement d'estimer la répartition des masses moléculaires, mais aussi de juger de l'efficacité éventuelle de son couplage avec la chromatographie sur SE-Sephadex pour la purification de certains composants.

Ainsi, les analyses électrophorétiques en gel d'acrylamide nous ont été précieuses dans l'estimation du degré de purification obtenu. Bien que ce type de fractionnement montre des possibilités telles qu'il mérite un intérêt certain, il faut constater que le rendement obtenu (75 %) devrait pouvoir être amélioré.

La présence de grosses quantités de sel jointe à l'existence dans les venins d'une forte proportion de substances de taille relativement faible, crée des difficultés expérimentales. De fait, dans les fractionnements de venins du type *Naja* et principalement dans le cas du *Naja naja atra*, la dessalination des fractions obtenues reste l'un des problèmes principaux.

La dialyse est exclue du fait de la trop forte proportion de molécules de faible poids moléculaire.

L'ultrafiltration au travers de membranes de collodion (limite de rétention: PM 1.000), bien que donnant des résultats satisfaisants, entraîne encore la perte de 7 à 10 % du venin et on constate parfois des précipitations sur la membrane. Il est à noter que les substances passant au travers de la membrane peuvent être récupérées en soumettant le venin total à une ultrafiltration dans l'eau préalablement à tout autre processus de fractionnement.

La dessalination par filtration sur gel fortement réticulé présente également des difficultés: on observe avec certains supports des absorptions irréversibles même lorsqu'on utilise des solutions d'élution à force ionique élevée. De plus, l'élution des sels est parfois trop proche de celle des protéines[30] pour obtenir une dessalination satisfaisante; ce phénomène est probablement dû à un entraînement des sels par une des protéines constituantes du venin[8].

L'utilisation d'un gradient de "sel volatil" (s'éliminant à la lyophilisation) constitue une importante simplification de la technique. Elle diminue considérablement les manipulations et est de ce fait beaucoup moins traumatisante.

Bien que donnant des résultats encourageants le résolution obtenue par ce mode d'élution n'est cependant pas encore aussi bonne que celle donnée par le tampon

phosphate et le gradient NaCl. Des essais en ce sens sont actuellement poursuivis dans notre laboratoire.

REMERCIEMENTS

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RÉSUMÉ

Le venin de *Naja naja atra* a été fractionné en quatorze fractions bien distinctes par chromatographie sur sulphoéthyl-Sephadex. La composition de ces fractions est étudiée. Un certain nombre d'activités enzymatiques ont été mesurées: la phospholipase A, la cholinestérase, la L-amino acide oxydase, l'adénosine triphosphatase, la 5'-nucléotidase, la ribonucléase, la phosphodiestérase, la nicotinamide dinucléotide pyrophosphatase et l'hyaluronidase. Plusieurs inhibiteurs de processus enzymatiques ont été estimés (inhibition de la glycolyse anaérobie, inhibition du système cytochrome oxydase et inhibition de l'acétylcholinestérase). Certaines activités biologiques, comme la toxicité, le pouvoir anticoagulant, la cytotoxicité, le facteur lytique direct sont également décrites. Les électrophérogrammes en gel d'acrylamide sont donnés pour les fractions les mieux séparées; les répartitions de tailles moléculaires dans ces fractions ont été estimées par la technique de la chromatographie sur gel de dextrane.

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CHROM. 3680

UNTERSUCHUNGEN ZUR QUANTITATIVEN RADIODÜNNSCHICHT-CHROMATOGRAPHIE TRITIUMMARKIERTER VERBINDUNGEN

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SUMMARY

Investigations on the quantitative radio thin-layer chromatography of tritium-labelled compounds

A measuring arrangement for the quantitative radio thin-layer chromatography of tritium and other low-energy β-emitters is described. The influence of various chromatographic and technical factors on the efficiency of the measurement and the reproducibility of quantitative results is investigated. At a detection limit of 2 nC the measuring efficiency for tritium was 1.5 % (^{14}C: 0.1 nC, 23.5 %). The over-all error of the method ranges from 10 to 20 %, depending on the level of activity applied.

Aus der Kombination radioanalytischer und chromatographischer Systeme hat sich neben anderen Verfahren besonders die Radiodünnschichtchromatographie energiearmer β-Strahler infolge ihres breiten Anwendungsspektrums auf dem Gebiet biologisch-medizinischer Tracer-Untersuchungen zu einer erfolgversprechenden Methode entwickelt. Einen Überblick über die bisher bestehenden apparativen und messtechnischen Möglichkeiten gibt SNYDER[1].

Nach Untersuchungen von SCHULZE UND WENZEL[2–4] sind zur stufenweisen oder kontinuierlichen Tritiummessung auf Dünnschichtchromatogrammen bevorzugt offene Gasdurchflusszählrohre geeignet, da in dieser Anordnung das empfindliche Zählvolumen durch den Kollimatorschlitz bis auf die Chromatogrammoberfläche geführt werden kann und damit weitgehend eine quantitative Erfassung der aus dem Chromatogramm emittierten β-Strahlung ($E_{max.}$ = 18.5 keV) entweder als solche oder in Form negativer, sekundärer Ladungsträger gegeben ist. Angaben von BLEECKEN und Mitarbeitern[5], BOUCKE[6] und RAVENHILL UND JAMES[7] befassen sich darüber hinaus mit Fragen der Korrelation zwischen Fehlerbreite und messtechnischen Parametern[6], mit dem Einfluss der chromatographierten Substanzmenge auf den Wirkungsgrad der Tritiummessung[5] sowie mit speziellen apparativen Lösungen[5,7]. MOYE[8] fordert für ^{14}C- und ^{35}S-Messungen konstante Schichtdicken, gibt jedoch keinen Hinweis für ihre routinemässige Ausmessung vor der Aktivitätsmessung.

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In früheren Mitteilungen[9,10] haben wir bereits auf die Verwendungsmöglichkeit des Gasdurchflusszählrohres VA-Z-530* für radiodünnschichtchromatographische ^{14}C- und ^{3}H-Messungen hingewiesen. Hiervon ausgehend untersuchten wir mit Hilfe einer einfachen Messanordnung zur kontinuierlichen Messung und Registrierung der Aktivitätsverteilung dünnschichtchromatographisch getrennter tritiummarkierter Verbindungen verschiedene chromatographisch und messtechnisch bedingte Faktoren in ihrer Bedeutung für die Messeffektivität und Reproduzierbarkeit quantitativer Aussagen. Im Vordergrund standen hierbei bisher nicht oder nur unzureichend untersuchte Fragen zur Grösse abstandsbedingter Effektivitätsänderungen, zum Einfluss der Trägerschichtdicke auf die Messwertbildung sowie zur Bedeutung von Flächendifferenzen ^{3}H-führender Chromatogrammflecken für eine vergleichende Prüfung der für die quantitative Auswertung radiodünnschichtchromatographischer Analysen sich anbietenden Messgrössen. Über die Bildung elektrostatischer Oberflächenladungen[11], deren Charakteristik und Einfluss auf die Zählratenkonstanz und Fehlerbreite der Aktivitätsmessung sowie über die Eliminierung derartiger Aufladungseffekte bei tritiummarkierten Dünnschichtchromatogrammen haben wir an anderer Stelle berichtet[12].

METHODEN

Radiodünnschichtchromatograph

Allgemeiner Aufbau. Der Radiodünnschichtchromatograph (Fig. 1) besteht aus dem in seiner Zähldrahtanordnung modifizierten Gasdurchflusszählrohr VA-Z-530* und der Transporteinrichtung mit dem Chromatogrammschlitten. Den von BLEECKEN und Mitarbeitern[5] gegebenen theoretischen Erwägungen folgend, sowie im Sinne eines günstigen Kompromisses zwischen Auflösungsvermögen und Messeffektivität, verwendeten wir eine Schlitzblende ($l = 30$ mm) mit einer Spaltbreite von 2 mm. Zur Höhenverschiebung des Detektors dient ein Feintrieb in Verbindung mit einer stationären Messkala mit Noniusablesung. Als Voraussetzung für die Einstellung eines reproduzierbaren Minimalabstandes (bis 50 μm) zwischen Zählrohrblende und Chromatogrammoberfläche (Δ_{Bl-Ch}) erfolgt die routinemässige Ausmessung desselben ohne Beschädigung der Schichtoberfläche mittels der am Scanner stationär fixierten Messuhr durch indirekte Differenzmessung nach dem Schattenprinzip[13].

Die Standardabweichung der Einstellungswerte beträgt bei einem Abstand von Δ_{Bl-Ch} 0.5 mm $\pm$ 3.4 % ($n = 10$)**.

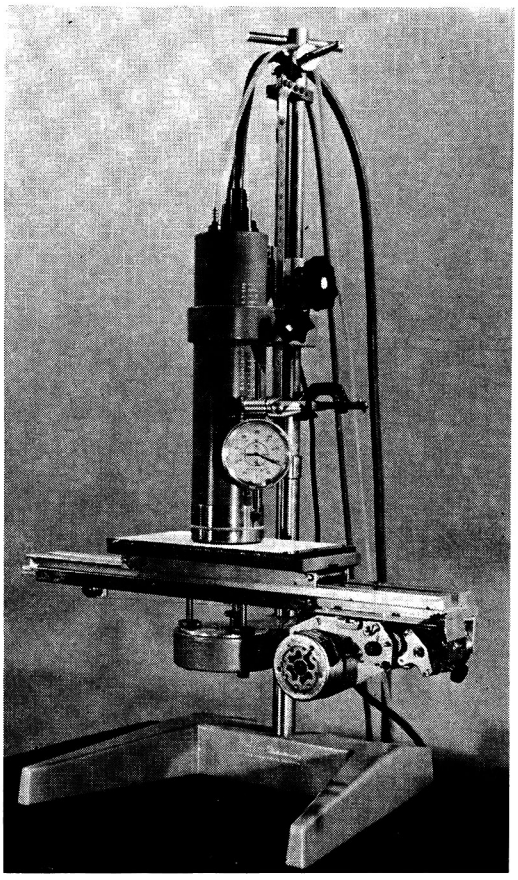
Der Detektor wird über den Strahlungsmessplatz VA-M-16D* (max. 4 kV) mit angeschlossenem Ergebnisdrucker betrieben. Die Registrierung erfolgt mit einem 10 mV eKB-Schreiber über den Impulsdichtemesser VA-D-41*. Die dem Startpunkt und der Lösungsmittelfront des vorliegenden Radiochromatogramms entsprechende Markierung der Aktivitätskurven erfolgt gemäss Fig. 2 automatisch durch die am Chromatogrammschlitten versetzbar angebrachten Magnetschalter B und C. Die am Schreibereingang den Messimpulsen parallel geschalteten Markierungsimpulse entsprechen einem Wert von *ca.* 0.5 mV. Die Markierungstaste A erlaubt eine von B und

* VEB Vakutronik Dresden.

** $s = \pm \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$.

C unabhängige zusätzliche Signierung der Schreiberkurven während des Messvorganges.

Transporteinrichtung zum Chromatogrammvorschub. Die Transporteinrichtung (Fig. 3) betreibt mittels Synchronmotor, angeflanschtem Getriebe (5) und Seiltrieb



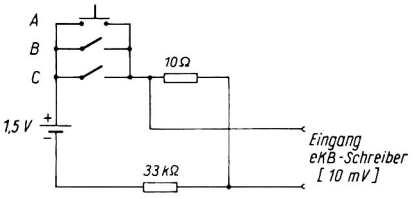


Fig. 1. Radiodünnschichtchromatograph (mit abgenommener Antriebsverkleidung und Ableitungsplatte).

Fig. 2. Schaltschema zur automatischen Markierung der Aktivitätsverteilungskurven.

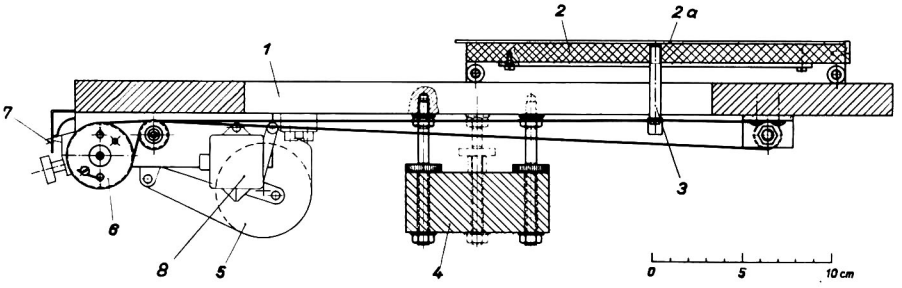


Fig. 3. Transporteinrichtung zum Chromatogramm-Vorschub (Erklärungen im Text).

(Nylon) den auf einer Metallschiene (1) laufenden, kugelgelagerten Chromatogrammschlitten (2). Die als Chromatogrammträger dienende 14 × 24 cm grosse Nickelplatte (2a) besitzt eine separate Erdableitung und verhindert durch ihren breitflächigen Kontakt mit der Chromatogrammunterseite eine positive elektrostatische Aufladung der Chromatogrammoberfläche und damit eine partielle oder totale Eliminierung der negativen, sekundären Ladungsträger[12]. Durch Verwendung eines dem Bandschreiber analogen Antriebsaggregates (Synchronmotor und Zahnradgetriebe) und Übereinstimmung des Durchmessers der Seilantriebsrolle (6) mit der Papiertransportrolle des eKB-Schreibers, entsprechen die fünf möglichen Vorschubgeschwindigkeiten '*c*' (20–3600 mm/h) der Transporteinrichtung denen des Bandschreibers. Aus praktischen und messtechnischen Gründen erwiesen sich *c*-Werte von 60, 200 und 600 mm/h am günstigsten. Die Schaltung der gewünschten Transportgeschwindigkeit und die Getriebeausrastung (Freilauf) erfolgt über (7), die automatische Transportabschaltung über den Endausschalter (8) nach Andruck des Mitnehmerstiftes (3). Vergleichende Messungen zur Kontinuität des Chromatogrammvorschubes ergaben für die drei genannten *c*-Bereiche bei einer Messtrecke von 10 cm maximale Abweichungen der Einzelwerte ($n = 10$)* von $< \pm 0.15\,\%$ und für 1 mm-Einheiten lediglich einen Wert von $s_{max.} = \pm 0.8\,\%$**. Die justierbare Dreipunktlagerung der Transporteinrichtung (4) erlaubt eine exakte Parallelität zwischen dem Blendenabschluss des Detektors und der Chromatogrammoberfläche.

Zähldraht-Form und -Charakteristik. Als Korrektur des kommerziellen Detektors machte sich eine Modifizierung der Zähldrahtanordnung erforderlich (Fig. 4). Von MÜNZEL UND HOLLSTEIN[14] liegen zu dieser Frage für stationäre Messungen eingehende Untersuchungen vor. Die Originalschleife (1) wurde durch einen in 4 mm

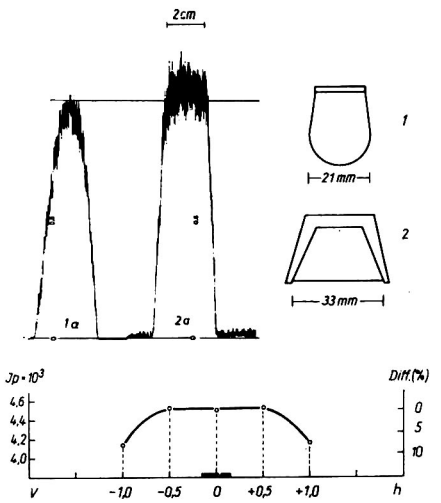


Fig. 4. Zähldraht-Form und Charakteristik der effektiven Messbreite von (2) (Aufnahme einer ^{3}H-Quelle parallel zur Blendenöffnung; c = 200 mm/h). 1 = Originalschleife; 2 = modifizierte Anordnung der Anode.

* $s = \pm \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$.

** Bei Verwendung eines Zahnstangenantriebes statt der Bandübertragung erhöhen sich die 1 mm-Werte um den Faktor 8–10.

Abstand zur Blendeninnenseite auf seiner Gesamtlänge parallel zur Blendenöffnung verlaufenden Zähldraht ((2), Molybdän, 30 μm Durchmesser) ersetzt. Die Aufnahme der Charakteristik beider Formen durch Vorschub einer ^{3}H-Quelle parallel zum Spalt zeigt, dass innerhalb der vorgegebenen Messbahn hierdurch eine wesentliche Verbesserung der optimalen Messbreite von ca. 0.3 (1a) auf 1.5 cm (2a) erzielt werden kann. Eine detaillierte Ausmessung der Impulsabweichungen vom Zähldrahtmittelpunkt ergab für $\pm$ 0.5 cm vollständige Konstanz und für $\pm$ 1.0 cm einen beidseitig gleichmässigen Abfall um ca. 8 %.

Zählrohrcharakteristik. Die stationär und bei einem Chromatogrammvorschub von 200 und 600 mm/h in Abhängigkeit vom Methanfluss und dem Abstand zwischen Blende und Chromatogrammoberfläche (Δ_{Bl-Ch}) aufgenommene Plateauentwicklung gibt Fig. 5 wieder. Als Vorlage dienten chromatographierte ^{3}H-Flecken mit einem Durchmesser von 7 mm. Während bei einem konstanten Δ_{Bl-Ch} von 0.5 mm Methangeschwindigkeiten zwischen 1 und 3 l/h keinen wesentlichen Einfluss auf Länge und Anstieg des Plateaus ausüben, bewirken Abstandsänderungen (CH_4: 2 l/h) von 0.3 auf 1.0 mm, abgesehen von der damit verbundenen und besonders für Δ_{Bl-Ch} = 1.0 mm offensichtlichen Effektivitätsänderung, einen Anstieg der Plateauwerte von 2.7 auf 6.0 %/100 V. Für eine Arbeitsspannung von U_A = 3.0 kV (Eingangsempfindlichkeit 2 mV), einer Methanbegasung von 2 l/h und Δ_{Bl-Ch} 0.5 mm beträgt bei einer Plateaulänge von 500 V der Plateauanstieg 3.7 %/100 V. Der Nulleffekt, der auf den erreichbaren Empfindlichkeitsgrad der Messanordnung einen wesentlichen Einfluss ausübt, liegt auch nach mehrjährigem Betrieb des Scanners bei < 35 Impulsen pro Minute.

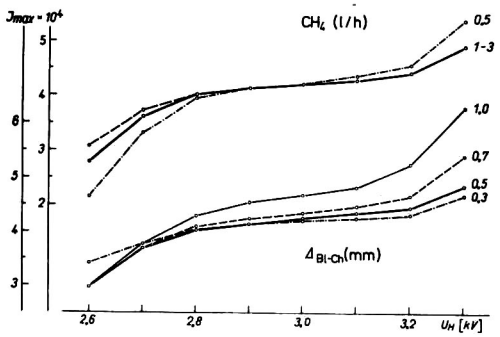


Fig. 5. Zählrohr-Charakteristik in Abhängigkeit von der Methanbegasung und dem Abstand Zählrohrblende–Chromatogrammoberfläche (Δ_{Bl-Ch}).

Dünnschichtchromatographie

Als Adsorbens fand Kieselgel G Merck Verwendung. Die Schichten wurden in üblicher Weise mit einem Streichgerät[15] aufgebracht und die Platten (10 × 20 cm) nach einstündiger Trocknung bei 105° unter Feuchtigkeitsausschluss aufbewahrt. Abgesehen von den Untersuchungen zum Einfluss der Trägerschichtdicke, betrug die Stärke der getrockneten Kieselgelschichten 200 μm $\pm$ 5 %. Die Dickenmessung erfolgte routinemässig vor der Verwendung der DC-Platten durch Differenzmessung unter Verwendung einer stationären Messuhr[13]. Um in allen Fällen eine gleichmässige und reproduzierbare Verteilung des markierten Substrates in der Trägerschicht und damit an der Oberfläche zu gewährleisten, wurden keine Punktauftragungen ver-

wendet, sondern sämtliche Untersuchungen nur an bereits chromatographierten DC-Platten vorgenommen. Als tritierte Verbindung fand Digitoxin-(u-^{3}H) (Lit. 9) mit einer spezifischen Aktivität von 182 mC/mMol und für die Aufstellung einer ^{14}C-Vergleichseichkurve Digitoxin(23-^{14}C) (11.2 mC/mMol[16]) Verwendung. Mit Ausnahme der Untersuchungen zum Spray-Einfluss (Spray: $SbCl_3$, 20%ig in Chloroform[17]; Trocknung: 3 min/120°), erfolgte die Aktivitätsmessung der mit Äthylacetat–Methanol (95:5) entwickelten Chromatogramme ohne vorherige Anfärbung.

EXPERIMENTELLE UNTERSUCHUNGEN

Wertigkeit der Messgrössen I_{max}, F_b, I_p und Einfluss der Fleckengrösse

Während für qualitative radiodünnschichtchromatographische Analysen die Art der verwendeten Messgrösse von untergeordneter Bedeutung ist, übt bei quantitativen Messungen die Wahl des Auswertungsmodus einen entscheidenden Einfluss auf die Messwertbildung aus. Zur Auswertung der Aktivitätsverteilungskurven stehen folgende drei Messgrössen zur Verfügung:

$I_{\max.}$ = maximale Impulshöhe des Peaks (Imp. $\times$ min^{-1});
F_b = Peakfläche F (mm^2) unter Berücksichtigung des Integrationsbereiches "B" ($F_b = F \times B \times 10^{-3}$);
I_p = gemessener Gesamtimpulswert pro Peak (Imp.).

I_p entspricht in seiner Entwicklung F_b, steht jedoch abweichend von F_b in Abhängigkeit von der vorgegebenen Messzeit und damit von der Geschwindigkeit des Chromatogramm-Vorschubes "c". Für Vorschubwerte im Bereich von 60–600 mm/h ist die zwischen I_p und c zu fordernde Proportionalität gegeben. Die Ermittlung von F_b erfolgt planimetrisch.

Eine vergleichende Wertung der Messgrössen $I_{\max.}$ und F_b kann eindeutig durch die Ausmessung chromatographierter ^{3}H-Aktivitätsflecken mit konstanter absoluter und spezifischer Aktivität jedoch differierender flächenmässiger Ausbreitung erfolgen (Fig. 6). Während $I_{\max.}$ bei einer Zunahme des Fleckendurchmessers von 5 auf 21 mm auf 25 % seines Ausgangswertes abfällt, verhält sich die Peakfläche F bzw. F_b unabhängig von der vorgelegten Chromatogrammfläche. Da die Grösse des Fleckendurchmessers infolge verschiedener chromatographischer Faktoren wie System-Zusammensetzung, Temperatur und Substanzmenge[5] als sehr variabel anzusehen ist und im Extremfall auch mit Verwaschungen und Schwanzbildungen gerechnet werden muss, kann eine Konstanz der flächenmässigen Ausbreitung der

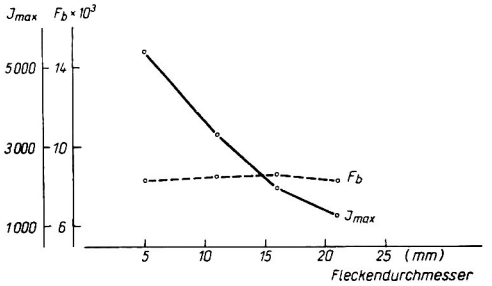


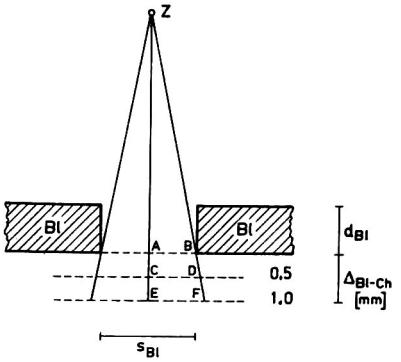
Fig. 6. Einfluss der Fleckengrösse der ^{3}H-Ablagerungen auf die quantitative Auswertung mittels der Messgrössen $I_{\max.}$ und F_b.

Flecken nicht vorausgesetzt werden. Damit scheidet für praktische Scanner-Messungen $I_{max.}$ als reale und reproduzierbare Messgrösse aus. Seine Verwendung erscheint nur bei vergleichenden methodischen Sondierungen ein und desselben Chromatogrammfleckes statthaft. Zur Auswertung aller sonstigen radiodünnschichtchromatographischen Analysen kann nur die Peakfläche F_b, bzw. nach entsprechender Eichung auch I_p, herangezogen werden.

Abstandsbedingte Effektivitätsänderungen

Die aus der Chromatogrammoberfläche emittierten β-Teilchen gehen praktisch verlustlos entweder als solche oder als negative, sekundäre Ladungsträger in die Registrierung ein. Die Limitierung der Messausbeute ist aus diesem Grunde fast ausschliesslich durch Faktoren der Selbstabsorption sowie der β-Absorption innerhalb der Trägerschicht gegeben. Hieraus resultiert, dass der Abstand Anode–Zählrohrblende (Fig. 7; $\overline{ZA}$ minus $d_{Bl} = 4$ mm) für die Frage der Messeffektivität von untergeordneter Bedeutung ist und lediglich in Korrelation mit der Grösse der Blendenöffnung den Ausfallswinkel der Feldlinien bestimmt. Messungen an Festpräparaten mit Abständen zwischen Anode und ^{3}H-Quelle im Bereich von 5–15 mm unterstreichen diese Feststellung durch eine Effektivitätsbewegung von nur 0.9 %/mm.

Im Gegensatz zu bereits vorliegenden Angaben unter Verwendung von Punktauftragungen[5,7] prüften wir die Frage abstandsbedingter Effektivitätsänderungen unter Bedingungen normaler Scanner-Messungen, d.h. unter Vorlage chromatographierter ^{3}H-Flecken mit einem der 3- bis 4-fachen Blendenspaltbreite entsprechenden Durchmesser (6–8 mm). Unter der Voraussetzung eines geradlinigen Verlaufes der von der Anode (Z) ausgehenden Feldlinien, einer optimalen Methansättigung sowie einer zentralen Anordnung des ^{3}H-Fleckes unter der Spaltblende lassen sich gemäss Fig. 7 die durch Variierung des Abstandes bedingten Effektivitätsverschiebungen auf eine Änderung des von den Feldlinien erfassten Ausschnittes der Chromatogrammoberfläche (mit konstanter ^{3}H-Beladung) zurückführen. Somit muss sich entgegen bisherigen Befunden[5,7] eine Abstandsvergrösserung in eine Erhöhung der



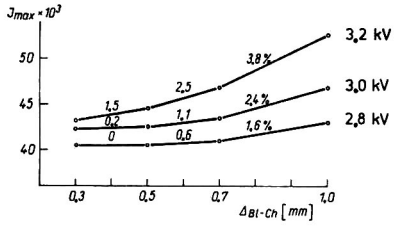


Fig. 7. Schematische Darstellung zum Feldlinienverlauf in Abhängigkeit von Anoden-Position, wirksamer Blendenweite und Chromatogramm-Abstand. Z = Anode; Bl = Zählrohrblende; s_{Bl} = Spaltbreite; d_{Bl} = Blendenstärke; $\Delta_{Bl\text{-}Ch}$ = Abstand Blende–Chromatogrammoberfläche.

Fig. 8. Abstandsbedingte Entwicklung der Messeffektivität in Abhängigkeit von der Zählrohrspannung unter Darstellung der prozentualen Impulsratenbewegung (%/0.1 mm $\Delta_{Bl\text{-}Ch}$).

registrierten Impulsrate umsetzen*. Bei einer Blendenspaltbreite von $s_{Bl} = 2$ mm und $\overline{ZA} = 5$ mm ($d_{Bl} = 1$ mm) lässt sich für eine Abstandsvergrösserung von Δ_{Bl-Ch} 0.5 → 1.0 mm mit Hilfe des Strahlensatzes ein Zuwachs der von den Feldlinien erfassten ^{3}H-führenden Chromatogrammfläche von 9.1 % berechnen.

Messungen zur Effektivitätsentwicklung (Fig. 8) zeigen die erwartete, in ihrer Steilheit vom Gasverstärkungsfaktor abhängige und parallel zur Abstandsvergrösserung verlaufende Steigerung der Impulsausbeute. Der Mittelwert des Gesamtanstiegs von Δ_{Bl-Ch} 0.5 auf 1.0 mm in dem plateaubegrenzenden Bereich von 2.8–3.2 kV entspricht mit einer Effektivitätserhöhung von 10.6 % (für U_A (3.0 kV) = 9.4 %) annähernd dem berechneten Wert.

Aus der dargestellten Impulsratenbewegung (prozentuale Impulsänderung/0.1 mm; Fig. 8) ergibt sich ferner, dass unkontrollierte Abstandsänderungen bei $\Delta_{Bl-Ch} >$ 0.7 mm ($U_A = 3.0$ kV) die quantitative Auswertung der Aktivitätsmessungen mit Abweichungen von 2.4% pro 0.1 mm (!) Abstandsdifferenz belasten. Da die entsprechenden Werte für Abstände unter 0.7 mm mit maximal 1.1% bzw. 0.2 %/0.1 mm wesentlich niedriger liegen, kann aus diesen Befunden die Forderung nach einer Verwendung reproduzierbarer Minimalabstände von $<$ 0.7 mm sowie die Notwendigkeit einer vor jeder quantitativen Aktivitätsmessung erforderlichen Abstandsmessung als Voraussetzung einer gleichbleibenden Abstandseinstellung abgeleitet werden.

Trägerschichtdicke

Unter der Voraussetzung einer gleichmässigen und reproduzierbaren ^{3}H-Substratverteilung in der Trägerschicht, muss auf der Basis der für Tritium ($E_{max} =$ 18.5 keV) charakteristischen Sättigungsdicke von 0.82 mg/cm^2 entgegen bisherigen Angaben[5,6] auch für den hier interessierenden analytischen DC-Bereich mit einer eindeutigen Einflussnahme der Trägerschichtdicke (d) auf die Messausbeute gerechnet werden. Messungen in zwei Aktivitätsniveaus (56 und 280 nC) beweisen für Schichtänderungen des Trägermaterials Kieselgel G im Bereich von 75–300 μm (d 100 μm ≈ 5 mg/cm^2) die zu erwartende und in beiden Niveaus parallel verlaufende Abhängigkeit des Flächen—bzw. Impulswertes von der Trägerschichtdicke (Fig. 9). Bei konstanter

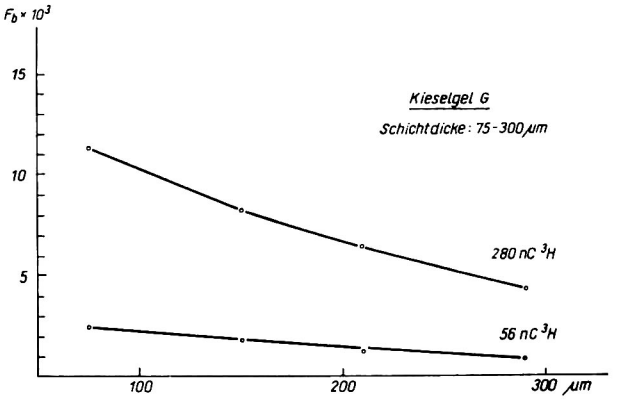


Fig. 9. Einfluss von Schichtdickenveränderungen (Kieselgel G; d = 75–290 μm) auf die Messeffektivität bei konstanter Ausgangsaktivität (56 und 280 nC).

* Dies gilt bis zu einem Limit, das unter den beschriebenen Messbedingungen bei Δ_{Bl-Ch} 1.5 mm liegt.

absoluter und spezifischer Ausgangsaktivität bedingt ein Anstieg der Schichtdicke von 100 auf 290 μm einen durchschnittlichen Abfall der Messeffektivität auf 39.3 % (56 nC: 38.1 %; 280 nC: 40.6 %).

Aus der auf der Grundlage dieser Werte zu berechnenden hohen Effektivitätsbewegung von ca. 3.2%/10 μm d für d = 100–290 μm ergibt sich hinsichtlich der Fehlerbreite quantitativer Messungen die Schlussfolgerung, dass die Vorlage konstanter Schichtdicken eine der wesentlichsten Voraussetzungen für den Erhalt reproduzierbarer Aussagen darstellt. Die gezeigte Entwicklung gilt nur für Tritium und Kieselgel G und kann in ihrem quantitativen Verlauf infolge des spezifischen Charakters der Sättigungsdicke und des Flächengewichtes nicht auf andere Nuklide oder Trägermaterialien[6] übertragen werden.

Aktivitätsbestimmung und Fehlerbreite der Methode

Die bei konstanter spezifischer Aktivität vorgenommene Tritiumeichung erfolgte bei einer Trägerschichtdicke von d = 200 μm und unter Berücksichtigung der genannten messtechnischen und chromatographischen Voraussetzungen über einen Bereich von 5.6–280 nC (Fig. 10; ^{3}H (a)). Die der Eichkurve zugeordnete Effektivitätskonstante K_{eff} (^{3}H (a): 24.4) entspricht dem Quotienten

$$K_{\text{eff}} = \frac{F_b}{A\,[\text{nC}]}$$

und stellt damit ein Mass für die unter den vorgegebenen Bedingungen erreichbare Effektivität der Nuklide dar. Eine Nachbehandlung der entwickelten Chromatogramme durch Spray mit Antimontrichlorid und anschliessender Trocknung bei 110° bis zum Eintritt der Farbreaktion bewirkt ein Absinken des K_{eff}-Wertes auf 17.0

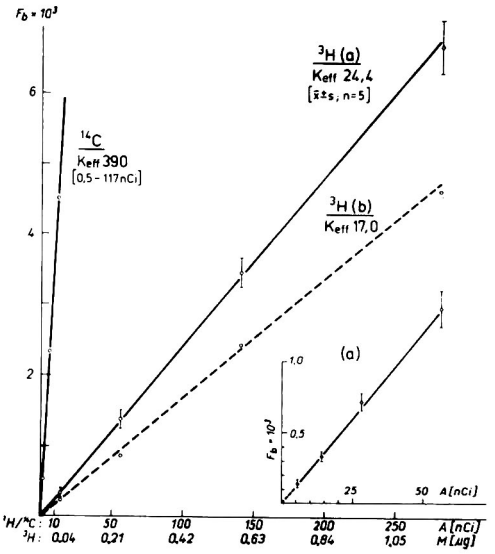


Fig. 10. Eichkurven zur radiodünnschichtchromatographischen Aktivitätsmessung von Tritium (5.6–280 nC) und Kohlenstoff-14 (0.5–117 nC); planimetrische Auswertung als F_b. ^{3}H (a) = Chromatogramme ohne Nachbehandlung; ^{3}H (b) = Chromatogramme nach Anfärbung mit $SbCl_3$; ^{14}C = Chromatogramme ohne Nachbehandlung.

(^{3}H (b)). Für die vergleichsweise über einen Bereich von 0.5–117 nC aufgenommene ^{14}C-Eichkurve beträgt K_{eff} 390. Die absolute Aktivität (A) in nC berechnet sich nach

$$A = \frac{F \times B \times 10^{-3}}{K_{eff}}$$

Die spezifische Aktivität der hier verwendeten Cardenolidtridigitoxoside kann im Bedarfsfall im Anschluss an die Aktivitätsmessung kolorimetrisch durch Reaktion der aus den aktivitätsführenden Banden gewonnenen Eluate mit 2,4,2',4'-Tetranitrodiphenyl bestimmt werden[18].

Die maximale, planimetrisch noch einwandfrei auswertbare Empfindlichkeit der Messanordnung liegt für Tritium bei 2 nC und für Kohlenstoff-14 bei 0.1 nC. Auf der Grundlage der I_p-Werte (c = 200 mm/h) ergibt sich für Tritium eine Messeffektivität von 1.5 % und für Kohlenstoff-14 von 23.5 %. Unter den optimalen Bedingungen der Eichung betragen die relativen Standardabweichungen der Einzelwerte* für Tritium bei $n = 5$ für 5.6 nC $\pm$ 14.5 %, 56 nC $\pm$ 7.9 % und für 280 nC $\pm$ 5.7 %.

Eine in Hinblick auf routinemässige Scanner-Messungen vorgenommene differenzierte Fehlereinschätzung zeigt, dass bei Einhaltung der beschriebenen messtechnischen Voraussetzungen der apparativ bedingte Fehler der reinen Impulsregistrierung auch im Fall einer Neueinstellung bei Wiederholungsmessungen des gleichen Dünnschichtchromatogramms unter 5 % liegt. Durch Einbeziehung des dünnschichtchromatographischen Teiles der Methode, in den ausser den bereits genannten Faktoren die geminderte Auftragungsgenauigkeit im μl-Bereich (incl. Pipettenfehler) eingeht, sowie unter Berücksichtigung der planimetrischen Auswertung mit relativen Standardabweichungen von $\pm$ 2–4 % ($n = 5$), ergibt sich ein durchschnittlicher Gesamtfehler der Methode von $\pm$ 10–20 %, der entsprechend den der Eichkurve zugeordneten Abweichungen je nach vorliegendem Aktivitätsniveau zur oberen oder unteren Grenze tendiert.

ZUSAMMENFASSUNG

Es wird über eine Messanordnung zur quantitativen Radiodünnschichtchromatographie von Tritium und anderen energiearmen β-Strahlern sowie über Untersuchungen zum Einfluss verschiedener chromatographisch und messtechnisch bedingter Faktoren auf die Messeffektivität und Reproduzierbarkeit quantitativer Aussagen berichtet. Bei einer Nachweisgrenze von 2 nC beträgt die Messeffektivität für Tritium 1.5 % (^{14}C: 0.1 nC, 23.5 %). Der Gesamtfehler der Methode bewegt sich je nach vorgegebenem Aktivitätsniveau zwischen 10 und 20 %.

DANK

Fräulein S. JÜNGLING und Herrn W. HOSENFELDER bin ich für ihre wertvolle Mitarbeit sehr zu Dank verpflichtet.

* $s = \pm \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$.

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CHROM. 3682

CLASSIFICATION OF CRUDE ANTIBIOTICS BY INSTANT THIN-LAYER CHROMATOGRAPHY (ITLC)

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SUMMARY

We have presented a thin-layer chromatographic method for identifying antibiotics contained in a crude mixture during the early stages of isolation of these compounds. The method attempts to assess rapidly the probability that the antibiotic in question is an already known one. A total of 84 antibiotics was included in this study.

Used alone, this method will not identify an individual antibiotic in a crude mixture, but it will narrow the choice of identities to a small number. Additional chemical, physical, and microbiological testing are required to distinguish individual antibiotics.

The method is applicable to samples of crude antibiotics, which are themselves easily prepared. It does not require the parallel evaluation of standard samples of antibiotics.

INTRODUCTION

Much time and effort are wasted in identifying supposedly "new" antibiotics that then turn out to be already known. A simple, rapid means of identifying antibiotics is of great value.

Chromatographic analysis, which had earlier been used to identify such natural products as steroids and alkaloids, has been applied to the systematic identification of antibiotics.

The summarized paper chromatogram, obtained when the R_F values of antibiotics tested in several solvent systems are represented graphically, was employed by ISHIDA *et al.*[1]. When AMMANN AND GOTTLIEB[2] used this technique to characterize antifungal antibiotics, they found that it sometimes failed to differentiate between closely related antibiotics. SNELL *et al.*[3], who used the same technique to differentiate 19 polypeptide antibiotics that inhibit Gram-positive organisms, pointed out the danger of attaching any significance to the specific R_F values in summarized paper chromatograms obtained from samples of crude antibiotics. Their observation that other constituents of the culture medium were able to influence the rates of migration

of the antibiotics led them to suggest the use of solvent systems that would differentiate antibiotics by giving widely different rates of movement for each substance.

Both BRODASKY[4] and FANG *et al.*[5] employed paper chromatography to identify the individual components in mixtures of antibiotics. BRODASKY, working with three pure antibiotics, found that R_F values determined by this technique were more reproducible than those determined by silica gel thin-layer chromatography. FANG *et al.* used six solvent systems simultaneously to separate approximately 50 mixed antibiotics.

MIYAZAKI *et al.*[6] refined paper chromatographic analysis by adding a salting-out process to their technique for classifying antibiotics. BETINA[7] and BETINA AND NEMEC[8] characterized antibiotics by means of their ionic character and of their R_F values, revealed by use of paper chromatography with buffered solutions.

BETINA[9] also classified 62 antibiotics by employing four solvent systems for a primary analysis, which yielded five classes, and additional solvent systems for a secondary analysis, which yielded 14 subclasses.

IKEKAWA *et al.*[10] used color-forming reagents to locate macrolide antibiotics isolated on thin-layer chromatograms. BETINA AND BARATH[11] and BICKEL *et al.*[12] subsequently combined thin-layer chromatography with bioautography for the detection of antibiotics.

SCHUURMANS *et al.*[13] employed strains of mammalian cells to screen for antitumor activity several antibiotics previously isolated by paper chromatography and paper strip electrophoresis.

The essential characteristics sought in a method for identifying unknown antibiotics were rapidity and applicability to easily prepared samples of crude antibiotics. The elimination of parallel evaluation of standard samples of antibiotics, after the chromatographic system had been standardized in a particular laboratory, was considered a worthwhile goal.

The time-consuming preparation of thin-layer chromatographic plates was avoided by use of Eastman Chromagram sheets, which also require only a short time for development. The method to be described below proved applicable to samples prepared by three different techniques, all based on simple solvent extraction.

It was taken for granted that the method would not be useful in the case of antitumor substances that lack antibacterial activity and that additional chemical, physical, and microbiological testing would be required for final identification of the antibiotics.

MATERIALS AND METHODS

Solvent systems

Antibiotics were first analyzed in three solvent systems: α = methanol; β = 10% methanol in chloroform; γ = chloroform.

The four groups yielded by this initial analysis were divided into 15 subgroups by the use of 11 additional solvent systems (see Table I).

Development of chromatograms and detection of antibiotics

Eastman Chromagram sheets, silica gel type 6060, henceforth referred to as ITLC (instant thin-layer chromatography) sheets, were partially deactivated by

TABLE I

Group	*Solvent system*	
I	pyridine–water (1:1)	Ia
	pyridine–water–absolute ethanol (1:1:1)	Ib
	pyridine–water–absolute ethanol (1:1:3)	Ic
II	butanol–methanol (1:1)	IIa
	chloroform–methanol (1:1)	IIb
	absolute ethanol	IIc
III	methanol–benzene (12:88)	IIIa
	methanol–benzene (6:94)	IIIb
	methanol–benzene (4:96)	IIIc
IV	methanol–benzene (1:99)	IVa
	methanol–benzene–chloroform (1:49:50)	IVb

exposure to air at room temperature (50–65 % relative humidity) for 24 h prior to use. Single glass chambers, lined with filter paper, were used for development of the chromatograms. For each antibiotic, the solvent systems used for development are given in Tables III and IV.

After development, the chromatograms were scanned under ultraviolet light and then bioautographed. The usual incubation time was 18 h at 37°, after the dried chromatogram had been placed directly on a filter paper resting on the seeded agar.

Methods of preparing samples of crude antibiotics

Three different methods were employed for the preparation of samples of crude antibiotics obtained from the 19 model fermentations.

Preparation A. To 6 ml whole broth, 0.4 ml 6 *N* HCl was added in one test tube, 0.5 ml 2 *N* NH_4OH was added in a second, and no addition was made to a third. After the addition of 3 ml butanol to each tube, they were shaken for 15 min and centrifuged. From each tube, 0.02 ml of the supernatant was applied by micropipette to the ITLC sheet.

Preparation B. After the addition of 10 ml isopropanol to 6 ml whole broth, the test tube was shaken for 15 min and centrifuged. A total of 0.04 to 0.06 ml of the supernatant was applied by micropipette to the ITLC sheet.

Preparation C. Ten milliliters of the isopropanol solution described in Preparation B were dried *in vacuo*. The residue was triturated twice with 1 ml ethanol and the ethanolic solutions were combined.

To the ethanol-insoluble triturate, 2 ml water were added, followed by 2 ml acetone, leading to the precipitation of proteins. The resulting suspension was centrifuged and a total of 0.02 ml, drawn from the supernatant as well as from the earlier ethanolic solution, was applied by micropipette to the ITLC sheet.

Antibiotics

Standard samples of 84 antibiotics were dissolved, each in its proper solvent, to give 1 % concentrations. The volume of solution applied to the ITLC sheets ranged from 1 to 60 λ, depending on the level of activity of each antibiotic against the micro-

TABLE II

ANTIBIOTICS INCLUDED IN THE ITLC CLASSIFICATION STUDY

Antibiotic	*Volume of antibiotic solution [1% conc.] (λ)*	*Microörganism used for bioautography*
Actidione	4	*Saccharomyces cerevisiae*
Actinobolin	5	*Staphylococcus aureus*
Actinomycin C_2	2	*Staph. aureus*
Actinomycin C_3	2	*Staph. aureus*
Amicetin	10	*Staph. aureus*
Aminosidin	10	*Staph. aureus*
Amphomycin	5	*Staph. aureus*
Anisomycin	20	*S. cerevisiae*
Azacolutin	30	*S. pastorianus*
Bacitracin	10	*Staph. aureus*
Bostrycoidin	2	*Bacillus subtilis*
Candicidin	10	*Candida albicans*
Candidin	2	*C. albicans*
Celesticetin	10	*Staph. aureus*
Cephalothecin	5	*Staph. aureus*
Chartreusin	3	*Staph. aureus*
Chloramphenicol	1	*Staph. aureus, Escherichia coli*
Citrinin	10	*Staph. aureus*
Clavacin	3	*E. coli*
C-73	20	*C. albicans*
Dihydrostreptomycin	20	*E. coli*
DON	40	*Staph. aureus*
Duramycin	20	*B. subtilis*
Echinomycin	5	*Staph. aureus*
Erythromycin	3	*Staph. aureus*
Esperin	10	*Staph. aureus*
Etamycin	10	*Staph. aureus*
Filipin	30	*S. cerevisiae*
Fungimycin	2	*S. cerevisiae*
Fusanin B	10	*B. subtilis*
Fusarubin	4	*Staph. aureus*
Gentamycin D	3	*Staph. aureus*
Gentamycin C_1	3	*Staph. aureus*
Gentamycin C_2	3	*Staph. aureus*
Gramicidin S	5	*Staph. aureus*
Griseofulvin	60	*C. albicans*
Hamycin	10	*C. albicans*
Humatin	10	*E. coli*
Hygromycin B	10	*Staph. aureus*
Javanicin	10	*B. subtilis*
Kanamycin	5	*Staph. aureus*
Lincomycin	3	*Staph. aureus*
Lucensomycin	5	*S. cerevisiae*
Mitomycin	5	*Staph. aureus*
Mycostatin	10	*C. albicans*
Neomycin C	10	*B. subtilis*
Nocardorubin	1	*Staph. aureus*
Nogalamycin	1	*Staph. aureus*
Novobiocin	10	*Staph. aureus*
Oleandomycin	10	*Staph. aureus*
Oligomycin	1	*S. cerevisiae*
Oxytetracycline	1	*Staph. aureus*
Polymyxin B	30	*E. coli*
Prasinomycin	3	*Staph. aureus*

(*continued on p. 491*)

TABLE II (*continued*)

Antibiotic	*Volume of antibiotic solution [1% conc.] (λ)*	*Microörganism used for bioautography*
Psicofuranine	20	*Staph. aureus*
Puromycin	20	*Staph. aureus*
Rhodomycetin	2	*Staph. aureus*
Rhodomycin	3	*Staph. aureus*
Rimocidin	10	*S. cerevisiae*
Ristocetin	5	*Staph. aureus*
Rubiflavin	1	*Staph. aureus*
Saramycetin	10	*Paecilomyces varioti*
Septacidin	20	*Trichophyton mentagrophytes*
Spiramycin	40	*Staph. aureus*
Streptomycin	5	*Staph. aureus*
Streptothricin BII	10	*Staph. aureus*
Streptovaricin	10	*Staph. aureus*
Streptozotocin	5	*Staph. aureus*
Streptovitacin	40	*S. pastorianus*
Subtilin	10	*Staph. aureus*
Sulfocidin	5	*Staph. aureus*
Tetracycline	1	*Staph. aureus*
Thiolutin	3	*Staph. aureus*
Thiostrepton	5	*Staph. aureus*
Tyrothricin	10	*Staph. aureus*
Toyocamycin	10	*C. albicans*
Trichomycin	10	*Staph. aureus*
Tubercidin	5	*C. albicans*
Tylosin	5	*C. albicans*
Unamycin A	25	*S. cerevisiae*
Usnic acid	10	*B. subtilis*
Vancomycin	10	*Staph. aureus*
Vernamycin A	5	*Staph. aureus*
Viomycin	10	*Proteus vulgaris*

organism used for bioautography. Data on volumes and microorganisms used are given in Table II.

Buffered ITLC

The ionic character of various antibiotics was determined by chromatography on buffered ITLC sheets. Prior to application of the antibiotic samples, each sheet was immersed in 0.5 *M* phosphate buffer solution of pH 2 or pH 11, then dried in air. The chromatograms were developed with the least polar solvent in which the antibiotic still showed movement (see Table V). Development of the chromatogram was followed by standard bioautography.

RESULTS

The prime criterion employed in our method of classification was the occurrence of movement of an antibiotic in a specific solvent system. Analysis of 84 pure antibiotics with three primary solvent systems produced a scheme containing four primary groups: group I = antibiotics showing no motion in solvents α, β, or γ; group II = antibiotics moving only in α; group III = antibiotics moving in α and β,

TABLE III

R_F VALUES OF ANTIBIOTICS INCLUDED IN THE ITLC CLASSIFICATION STUDY

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]					
		α	*β*	*γ*	*Ia*	*Ib*	*Ic*
I-1							
	Gentamycin D	0	0	0	0	0	0
	Gentamycin C_1	0	0	0	0	0	0
	Gentamycin C_2	0	0	0	0	0	0
	Humatin	0	0	0	0	0	0
	Neomycin	0	0	0	0	0	0
I-2	(Ia)[b]						
	Aminosidin	0	0	0	0–0.8	0	0
	Dihydrostreptomycin	0	0	0	0.25	0	0
	Hygromycin B	0	0	0	0.1	0	0
	Streptomycin	0	0	0	0.15	0	0
	Streptothricin	0	0	0	0.3	0	0
	Viomycin	0	0	0	0.3–0.5	0	0
I-3	(Ia, Ib)						
	Kanamycin	0	0	0	0–0.3	0–0.2	0
	Rubiflavin	0	0	0	0–0.2	0–0.15	0
I-4	(Ia, Ib, Ic)						
	Polymyxin B	0	0	0	0.7	0.56	0.4
	Ristocetin	0	0	0	0.9	0.7	0.7
	Vancomycin	0	0	0	0.3–0.8	0.7	0–0.6
		α	*β*	*γ*	*IIa*	*IIb*	*IIc*
II-1							
	Candidin	0–0.32	0	0	0	0	0
	Gramicidin S	0–0.2	0	0	0	0	0
	Prasinomycin	0.28	0	0	0	0	0
	Duramycin	0.6	0	0	0	0	0
II-2	(α, IIa, or IIb)						
	Amphomycin	0.35	0	0	0.08	0	0
	Candicidin	0.4–0.6	0	0	0	0.3–0.4	0
	Mycostatin	0.5	0	0	0.22	0	0
II-3	(α, IIa, IIb)						
	Bacitracin	0.23	0	0	0.2	0.15	0
	Subtilin	0.7	0	0	0.5	0.2	0
	Trichomycin	0.5	0	0	0–0.15	0.1	0
	Unamycin A	0.5	0	0	0.3	0.2	0
II-4	(α, IIa, IIb, IIc)						
	Azacolutin	0.7	0	0	0.1	0.1	0.05
	Cephalothecin	0.8	0	0	0.6	0.6	0.6
	Hamycin	0–0.6	0	0	0–0.1	0–0.1	0.05
	Lucensomycin	0.4	0	0	0.22	0.12	0.06
	Oxytetracycline	0–0.4	0	0	0–0.4	0–0.25	0–0.2
	Rimocidin	0.5	0	0	0–0.3	0–0.3	0–0.15
	Septacidin	0.6	0	0	0.55	0.5	0.45
	Tetracycline	0–0.3	0	0	0–0.5	0–0.2	0–0.2

[a] See MATERIALS AND METHODS section for details.

[b] Designation in parentheses identifies the solvent systems in which the antibiotic moved.

(*continued on p.* 493)

TABLE III (*continued*)

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]					
		α	*β*	*γ*	*IIIa*	*IIIb*	*IIIc*
III-1	(α, β)						
	Fungimycin	0–0.75	0–0.2	0	0	0	0
	Novobiocin	0.71	0.7	0	0	0	0
	Oleandomycin	0.48	0.3	0	0	0	0
	Rhodomycetin	0.05	0.05	0	0	0	0
	Saramycetin	0.62	0.62	0	0	0	0
III-2	(α, β, IIIa)						
	Amicetin	0.6	0.28	0	0.1	0	0
	Citrinin	0.72	0.8	0	0.16	0	0
	Erythromycin	0.42	0.26	0	0.1	0	0
	Filipin	0.6	0.15	0	0.18	0	0
	Nogalamycin	0.46	0.4	0	0.3	0	0
	Rhodomycin	0.73	0.5	0	0.3	0	0
	Streptozotocin	0.6	0–0.4	0	0–0.2	0	0
	Toyocamycin	0.65	0.28	0	0	0.1	0
	Tubercidin	0.62	0.22	0	0.05	0	0
III-3	(α, β, IIIa, IIIb)						
	Anisomycin	0.5	0.35	0	0.2	0.1	0
	Fusanin B	0.65	0.58	0	0.2	0.1	0
	Lincomycin	0.41	0.25	0	0–0.2	0–0.1	0
	Puromycin	0.7	0.55	0	0.25	0–0.15	0
	Streptovitacin	0.41	0.25	0	0.23	0.1	0
	Sulfocidin	0.65	0.5	0	0.85	0–0.5	0
	Thiostrepton	0.62	0.56	0	0.25	0.15	0
	Tylosin	0.7	0.6	0	0.35	0.1	0
III-4	(α, β, IIIa, IIIb, IIIc)						
	Actidione	0.64	0.67	0	0.5	0.22	0.2
	Actinomycin C_2	0.82	0.8	0	0.5	0.26	0.05
	Actinomycin C_3	0.82	0.75	0	0.5	0.26	0.05
	Celesticetin	0.76	0.74	0	0.4	0.25	0.05
	Chloramphenicol	0.72	0.57	0	0.3	0.15	0.09
	Echinomycin	0.7	0.7	0	0.5	0.22	0.05
	Esperin	0.75	0–0.5	0	0.2	0.09	0.05
	Javanicin	0.7	0.68	0	0.72	0.71	0.71
	Mitomycin	0.72	0.48	0	0.25	0.1	0.05
	Nocardorubin	0.55	0.5	0	0.45	0.3	0.25
	Streptovaricin	0.79	0.78	0	0–0.5	0–0.4	0–0.4
		α	*β*	*γ*	*IVa*	*IVb*	
IV-1	(α, β, γ)						
	Actinobolin	0.42	0.2	0.08	0	0	
	DON	0.78	0.76	0.65	0	0	
	Psicofuranine	0.7	0.15	0–0.1	0	0	
	Spiramycin	0.6	0.4	0–0.3	0	0	
IV-2	(α, β, γ, IVa)						
	Etamycin	0.8	0.8	0–0.2	0.1	0	
	Oligomycin	0.43	0.43	0.32	0.2	0	
	Thiolutin	0–0.6	0.65	0.35	0.3	0	
	Vernamycin A	0.6	0.63	0.2	0.12	0	

(*continued on p.* 494)

TABLE III (*continued*)

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]				
		α	*β*	*γ*	*IVa*	*IVb*
IV-3	(α, β, γ, IVa, IVb)					
	Bostrycoidin	0.76	0.73	0.26	0.22	0.15
	Chartreusin	0.74	0.65	0.15	0.16	0.1
	Clavacin	0.7	0.65	0.3	0.25	0.16
	C-73	0.8	0.7	0.55	0.4	0–0.22
	Fusarubin	0.3	0.73	0.45	0.23	0.15
	Griseofulvin	0.75	0.75	0.52	0.5	0.25
	Tyrothricin	0.7	0.7	0.25	0.7	0.1
	Usnic acid	0.75	0.75	0.75	0.5	0.45

but not in γ; group IV = antibiotics moving in α, β, and γ. Of the 84 antibiotics tested, 16 were in group I, 19 in group II, 33 in group III, and 16 in group IV.

Application of 11 additional solvent systems to the members of the four primary groups yielded 15 subgroups. R_F values of all antibiotics in both the primary and secondary solvent systems are shown in Table III.

Nineteen of the 84 antibiotics tested were chosen as models and produced by fermentation. They included four from group I, five from group II, seven from group III, and three from group IV. Preparation methods A, B, and C (see MATERIALS AND METHODS) were applied to each of the 19 fermentation broths and all 57 resulting samples were tested in the ITLC system.

Table IV shows which methods of preparation yielded samples of these 19 antibiotics that could be satisfactorily applied to the ITLC sheets. It also gives the R_F values of the bioactive spots found in chromatograms of these compounds.

Thirty-six of the original 84 pure antibiotics were tested in the buffered ITLC system, as were 18 of the 19 crude antibiotic preparations named in Table IV. R_F values for compounds tested in the buffered ITLC system are given in Table V.

DISCUSSION

The purpose of this study has been to develop a screening technique that can reveal, within a few days, whether a microbiologically active principle found in a fermentation broth is an already known antibiotic. We worked with 84 known antibiotics. Because the method is partially dependent on bioautography, its application to the screening of antitumor antibiotics is limited to those in which antibacterial activity parallels *in vivo* antitumor activity. Such parallelism exists for about 85 % of antitumor antibiotics.

The method of SCHUURMANS *et al.*[13] for detecting antitumor antibiotics is based on the inhibition of cellular dehydrogenase activity and requires cultivation of mammalian cell strains under conditions that are sometimes difficult to achieve in a chemical laboratory. Like the method presented in this paper, it is not applicable to some antitumor antibiotics. It is, perhaps, of greatest value in the later stages of isolation of these compounds.

Our ITLC system, which combines the sequential analysis technique of SNELL *et al.*[3] with the simultaneous analysis technique of ISHIDA *et al.*[1], reduces the number

TABLE IV

R_F VALUES OF CRUDE PREPARATIONS OF ANTIBIOTICS IN THE ITLC SYSTEMS

Antibiotic	*R_F values[a] of bioactive spot[b] in specific solvent systems[c]*						*Types of crude preparation[c] successfully applied*
Group I	*α*	*β*	*γ*	*Ia*	*Ib*	*Ic*	
Dihydrostreptomycin	0 (0.5)	0	0	0.3 (0.4)	0	0	BC
Polymyxin B	0 (0.62)	0(0.53)	0 (0.62)	0.75	0.6	0.65	ABC
Rubiflavin	0	0	0	0–0.3	0–0.2	0	ABC
Streptomycin	0 (0.5)	0	0	0–0.15	0	0	BC
Group II	*α*	*β*	*γ*	*IIa*	*IIb*	*IIc*	
Azacolutin	0.75 (0)	0	0	0.14 (0)	0.12 (0)	0.05	ABC
Duramycin	0.7	0	0	0	0	0	ABC
Oxytetracycline	0.5	0	0	0–0.5	0–0.3	0–0.3	ABC
Prasinomycin	0.3	0	0	0	0	0	ABC
Tetracycline	0–0.4	0	0	0–0.5	0–0.5	0–0.3	ABC
Group III	*α*	*β*	*γ*	*IIIa*	*IIIb*	*IIIc*	
Actidione	0.64	0.7	0	0.5	0.23	0.2	ABC
Chloramphenicol	0.74	0.54 (0.57)	0 (0.3)	0.32 (0.45)	0.14 (0.35)	0.09 (0.28)	ABC
Erythromycin	0.43 (0.65)	0.28 (0.65)	0 (0.15)	0.1 (0.3)	0 (0.35)	0 (0.5)	ABC
Novobiocin	0.71	0.71 (0)	0	0	0	0	ABC
Saramycetin	0.62	0.6	0	0	0	0	ABC
Thiostrepton	0.62	0.6 (0)	0	0.28 (0)	0.13 (0)	0	ABC
Toyocamycin	0.65	0.3	0	0.1	0	0	ABC
Group IV	*α*	*β*	*γ*	*IVa*	*IVb*		
Bostrycoidin	0.76	0.73	0.28 (0.5)	0.25 (0.4)	0.15		ABC
Chartreusin	0.74	0.65	0.18	0.18	0.1		ABC
Vernamyacin A	0.6	0.63	0.1–0.25	0–0.12	0		ABC

[a] The R_F value in parentheses designates a minor second bioactive spot.
[b] Microörganisms used for bioautography are listed in Table II.
[c] See MATERIALS AND METHODS section for details.

TABLE V

R_F VALUES[a] OF ANTIBIOTICS IN BIOACTIVE SPOTS[b] IN A BUFFERED ITLC SYSTEM[c]

Antibiotic[d]	*Solvent system used to develop chromatogram*	*R_F values*	
		pH 2 buffer	*pH 11 buffer*
Actidione	β	0.61 (0.63)	0.61 (0.65)
Actinomycin C_2	β	0.71	0.75
Actinomycin C_3	β	0.70	0.75
Amicetin	β	0	0.3
Azacolutin	α	0.5	—
Bacitracin	α	0–0.65	0–0.1
Bostrycoidin	γ	0.4 (0.42)	0.55 (0.55)
Chartreusin	γ	0.5–0.7 (0.65)	0.1 (0.25)
Chloramphenicol	β	0.4 (0.45)	0.4 (0.43)
Citrinin	β	0.2	0
Dihydrostreptomycin	α	0	0.2 (0.25)
Erythromycin	β	0.4 (0.3–0.5)	0.58 (0.6)
Etamycin	β	0.8	0.8
Filipin	β	0.55	0.5
Fusarubin	γ	0.48	0.28
Griseofulvin	γ	0.4	0.4
Javanicin	γ	0.48	0.59
Mycostatin	α	0.5	0.45
Nogalamycin	β	0.36	0.44
Novobiocin	β	0.4 (0.42)	0 (0)
Oligomycin	α	0.26	0.26
Oxytetracycline	α	0–0.1 (0–0.2)	0.5 (0–0.6)
Polymyxin B	β	0–0.8 (0.65)	0.6 (0.5)
Prasinomycin	α	0.6 (0–0.6)	0.25 (0.2)
Rhodomycin	β	0–0.7	0.65
Rubiflavin	β	0 (0)	0–0.2 (0–0.2)
Saramycetin	α	0.57 (0.4–0.5)	0.3 (0.25)
Spiramycin	β	0.25	0.6
Streptomycin	α	0 (0–0.05)	0.3 (0.2–0.3)
Streptovitacin	β	0.45	0.45
Tetracycline	α	0–0.1 (0–0.18)	0–0.5 (0–0.54)
Thiostrepton	β	0.65 (0.68)	0.75 (0.75)
Toyocamycin	β	0–0.03 (0.05)	0.28 (0.3)
Tylosin	β	0.58	0.65
Unamycin A	α	0.45	0.35
Vernamycin A	γ	0–0.2 (0–0.22)	0–0.2 (0–0.2)

[a] R_F value in parentheses indicates a bioactive spot from the crude antibiotic preparation, in contrast to main value obtained from pure antibiotic.

[b] Microörganisms used for bioautography are listed in Table II.

[c] Amphoteric antibiotics may appear acidic, basic, or neutral in this two-buffer ITLC system.

[d] Same volume as shown in Table II.

of solvent systems required. The unknown antibiotic is classified as a member of a subgroup that contains only a few antibiotics. Use of a buffered ITLC system, as well as of additional microbiological and chemical tests, permits these few antibiotics to be distinguished from each other. Differentiation of antibiotics based on the characteristics revealed by such tests, as advocated by SNELL *et al.*[3], is more reliable than is differentiation based on the potentially misleading differences in R_F values. The only use made of R_F values in our system of classification is to demonstrate the movement of an antibiotic.

Examination of the data in Table III makes it apparent that the solvent systems employed have not placed into one subgroup all antibiotics with a close chemical relationship. This fact is both advantageous and disadvantageous. Antibiotics in any one subgroup that are not closely related chemically can be differentiated by chemical and microbiological means more easily than can those that are. On the other hand, mere assignment of an antibiotic to a subgroup does not reveal much about what type of antibiotic it is.

All three methods of preparing samples of crude antibiotics were, in general, equally useful. Method A failed with streptomycin and dihydrostreptomycin, both of which are insoluble in butanol. The inability of butanol to dissolve some antibiotics may be turned to advantage in screening a large number of fermentation broths. Method A may also give some information about the ionic character of the unidentified active principle. Method B, which was successful with all 19 model fermentations, required the application of only a single sample to each ITLC sheet. Method C, the most time-consuming one, yielded the purest samples and R_F values of these samples showed good reproducibility, even with variations in the fermentation procedure.

A crude preparation of an antibiotic does not give the same R_F values as does the pure form of the same substance (see Tables III and IV).

Bioautography of thin-layer chromatograms was described by BETINA AND BARATH[11], who employed a "fingerprinting" technique. BICKEL *et al.*[12] were able to place the thin-layer chromatogram, on a paper base, directly on to the seeded agar plate. We were able to place developed ITLC sheets on to a filter paper already resting on the seeded agar. Well-defined zones of inhibition were visible after the usual incubation period.

The present technique is inherently simple. An ITLC sheet is easy to handle. Chromatograms can be developed in a single glass chamber within 20 to 30 min, except for solvent systems containing pyridine, which require 3 to 4 h.

We have chromatographed antibiotics on columns, employing either silicic acid or silica gel deactivated in a manner similar to that used for the ITLC sheets. Our results have been in very good agreement with those obtained by means of the ITLC system.

On the ITLC sheets, some antibiotics streaked rather than moving as circular spots (Tables III, IV and V). Such streaking occurred in all solvent systems with aminosidin, fungimycin, gramicidin S, hamycin, kanamycin, oxytetracycline, and tetracycline. In other cases, like azacolutin, bacitracin, echinomycin, lincomycin, polymyxin B, rubiflavin, streptovaricin, sulfocidin, and vernamycin A, the streaking took place only in some solvents. The streaking behavior of an antibiotic may sometimes be used as an identifying characteristic.

The ITLC system does not require the use of standard samples of antibiotics for parallel evaluation after the system has been standardized in a particular laboratory. Results obtained by its use have shown good reproducibility. The initial step in identifying an unknown antibiotic is the determination of movement in specific solvent systems and the nature of the microbiological spectrum. Final identification depends on further chemical and microbiological tests.

Although the present study included only 84 antibiotics, application of the ITLC classification system to additional antibiotics is now in progress.

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

VII*. FURTHER INVESTIGATIONS OF DIRECT FLUOROMETRIC SCANNING OF AMINO ACID DERIVATIVES**

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SUMMARY

Direct estimation of DANS-, DNP- and PTH-amino acids separated on silica gel and polyamide layers, by means of fluorescence and fluorescence quenching techniques, is discussed. The reproducibility of different methods of peak measurement is compared. Special reference is made to the scanning of closely neighbouring spots.

Although thin-layer chromatography (TLC) has been well established in amino acid and peptide chemistry (for a review *cf.* refs. 3–6), there is very little known about the quantitative estimation of these compounds by means of *in situ* techniques. As far as we are aware, SEILER in Germany[7] was the first to use direct fluorometry for scanning of fluorescent spots of dimethylamino-naphthalenesulphonyl-(DANS)-amino acids. Independently and simultaneously, one of us described the direct fluorometric estimation of DANS-amino acids, as well as the application of the quenching technique in quantitative evaluation of dinitrophenyl (DNP)- and phenylthiohydantoin-(PTH)-amino acids[8]. In the meantime further investigations have been carried out, and beside silica gel layers, the use of instant polyamide layers according to WANG[9] has been studied. Our recent results are described in the present communication.

METHODS

Chromatography

TLC of *DANS-amino acids* was carried out on Silica Gel G layers (containing starch)[8] with benzene–pyridine–acetic acid (80:20:2, v/v)[10] or chloroform–methanol–

* For Part VI *cf.* ref. 1.
** 8th communication on "Application of thin-layer chromatography in sequence analysis of peptides." 7th communication *cf.* ref. 2.

acetic acid (75:20:5, v/v)[11]. Alternatively, instant polyamide layers[9*] and *e.g.*, heptane–*n*-butanol–acetic acid (3:3:1, v/v)[12] were used.

DNP-amino acids were chromatographed either on Silica Gel G layers (containing starch)[8] with chloroform–benzyl alcohol–acetic acid (70:30:3 v/v)[10] or with *n*-propanol–25 % ammonia (7:3, v/v)[10], or on polyamide sheets[9*] with benzene–acetic acid (4:1, v/v)[13].

Chromatograms of *PTH-amino acids* were carried out on Silica Gel G–zinc silicate layers (containing starch)[3,4,8] with chloroform–formic acid (100:5, v/v)[10] and chloroform–methanol(9:1, v/v)[10] or on polyamide layers[9*] with formic acid 90 %–water (1:1, v/v)[14].

Quantitative estimation

The spots were scanned, after drying the chromatoplates in a stream of cold air for exactly 30 min, by fluorometry using a Turner-Fluorometer 111 fitted with a door for thin-layer plates[**]. In some experiments the improved "de-luxe" door has been used[15**]. Speed of scan: 20 mm/min.

Conditions for scanning are given in Table I. The fluorometer units were recorded, generally at a voltage of 10 mV, with a Hi-Speed Recorder (W+W model 401)[***] normally using a speed of 8 cm/min.

TABLE I

CONDITIONS OF SCANNING[a]

Compound	*Layer*	*Excitation (nm)*	*1° filter*[b]	*2° filter*[b]	*Neutral density filter in addition to the 2° filter (%)*	*Sensitivity of fluorometer*[c]
DANS-amino acids	Silica gel	366	110–811	110–816	40 + 20	10 ×
DANS-amino acids	Polyamide	366	110–811	110–816	40 + 10	1 ×
DNP-amino acids	Silica gel	254	110–810	110–816	40	5 ×
DNP-amino acids	Polyamide	254	110–810	110–816	10	5 ×
PTH-amino acids	Silica gel with zinc silicate	254	110–810	110–816	20	1 ×
PTH-amino acids	Polyamide	254	110–810	110–816	10	5 ×

[a] Aperture to door in all experiments fully open (~ 3.4 mm). The samples were applied as spots, using 2 μl microcaps.
[b] Camag catalog nos.
[c] The blends 110–842 were used.

The scan was made perpendicular to the direction of chromatography. The layer was covered, except for a small strip, 12–14 mm, containing the spot under investigation, with a plain silica gel or polyamide sheet, respectively.

RESULTS

Our previous experiments on direct fluorometry[1,8,16–18] have been carried out with a prototype of the Camag/Turner Scanner. In the meantime a number of im-

* Cheng-Chin Trading Co. Ltd., No. 75 Sec. I. Hankow St., Taipei, Taiwan.
** Camag, Muttenz, BL, Switzerland.
*** Kontron AG, Zürich, Switzerland.

provements have been made[15,19] and therefore the working conditions had to be changed to some extent. In particular, the sensitivity could be kept considerably lower, as illustrated in Fig. 1. On the other hand, it was also found that the fluorescence peak area is dependent on the *sensitivity* of the fluorometer. The effect of using different fluorometers in scanning of spots is shown in Fig. 2. Fluorescence peaks of DANS-amino acids (Fig. 3) and quenching peaks of DNP-amino acids (Fig. 4) on silica gel and on polyamide layers show very clearly that such compounds can be measured with higher sensitivities on polyamide layers. The superiority of polyamide layers with respect to the sensitivity of scanning is partly compensated by the fact that the separation of amino acid derivatives is highly dependent upon the quality of polyamide used. Whilst the chromatographic resolution of amino acids

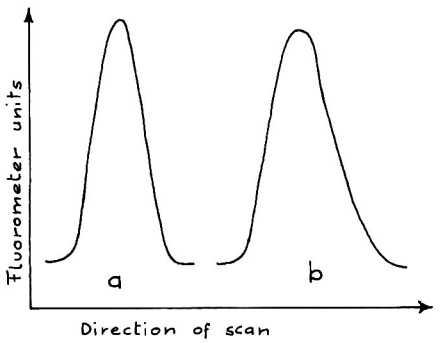

Fig. 1. Fluorescence peaks of DANS-proline on Silica Gel G. (a) 5 μg DANS-proline measured with the prototype (sensitivity: 10 ×, no neutral density filter was used). Peak area = 1420 mm^2. (b) 2 μg DANS-proline measured with the improved model (sensitivity: 10 ×, neutral density filters: 20 + 40%). Peak area = 1840 mm^2.

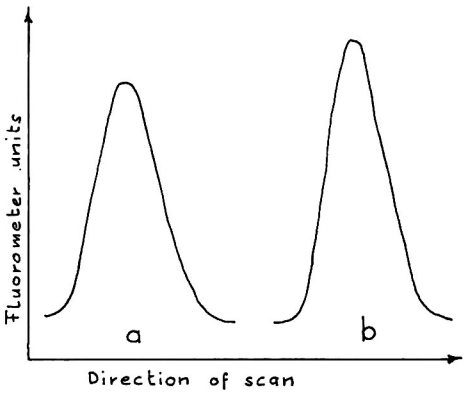

Fig. 2. Fluorescence peaks of DANS-proline on Silica Gel G. (a) 2 μg DANS-proline, measured with fluorometer I (sensitivity: 10 ×; neutral density filters: 20 + 40%). Peak area = 1840 mm^2. (b) 2 μg DANS-proline, measured with fluorometer II (sensitivity: 10 ×; neutral density filters: 20 + 40%). Peak area = 2040 mm^2.

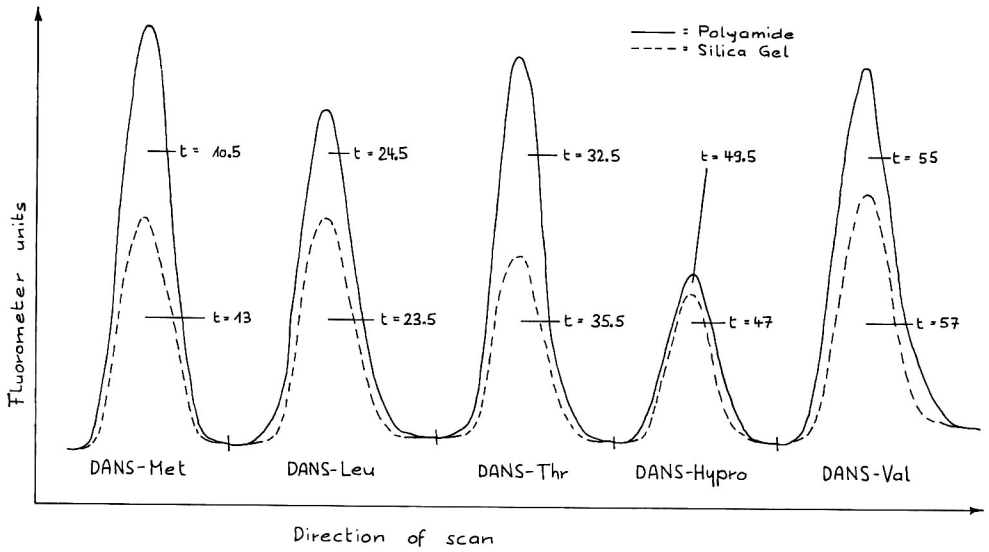

Fig. 3. Fluorescence peaks of DANS-amino acids (2 μg each) on Silica Gel G and polyamide, conditions are given in Table I. The higher sensitivity on polyamide layers is especially worthy of note. The "*t*-values" indicate the time (min) between plate drying and scanning (see METHODS).

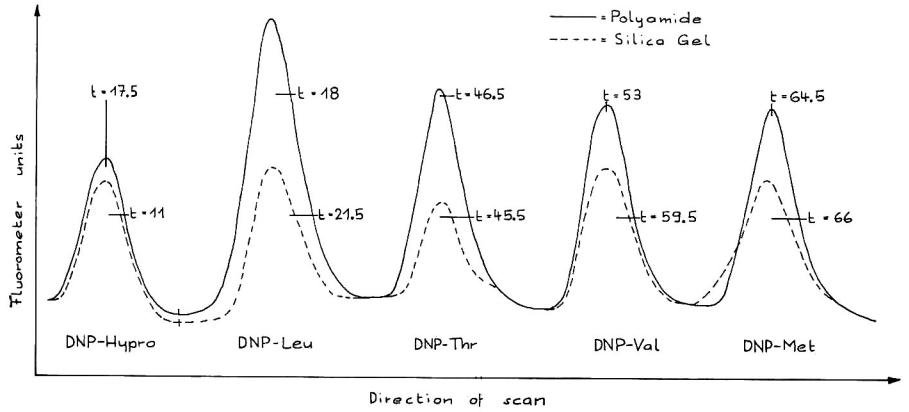

Fig. 4. Fluorescence quenching peaks of DNP-amino acids (2 μg each) on Silica Gel G and polyamide. The conditions are given in Table I. The higher sensitivity on polyamide layers is especially worthy of note. The "*t*-values" indicate the time (min) between plate drying and scanning (see METHODS).

on silica gel layers from different commercial suppliers seems to be hardly influenced by the quality of sorbent[20], the separation of DANS-, DNP- and PTH-derivatives is inferior using other types of polyamide. The sensitivity of scanning of PTH-amino acids on silica gel and polyamide is approximately of the same order of magnitude Fig. 5). The fluorescence peak areas of DANS-derivatives are given in Table II, while in Tables III and IV fluorescence quenching peak areas of DNP- and PTH-derivatives are given. These data may change with variation of the experimental conditions, or from one laboratory to another, but their relative values will serve as a guide line in

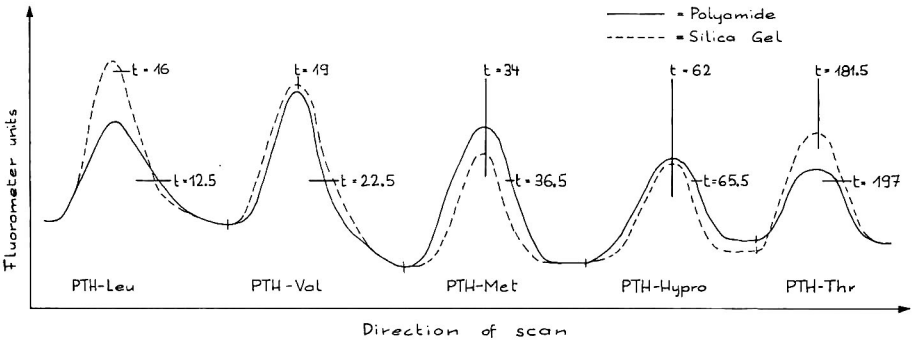

Fig. 5. Fluorescence quenching peaks of PTH-amino acids (2 μg each) on Silica Gel G (containing zinc silicate) and polyamide. The conditions are given in Table I. The "*t*-values" indicate the time (min) between plate drying and scanning (see METHODS).

practical work. It should be pointed out that in all cases of fluorescence quenching measurements, polyamide layers were capable of furnishing more constant baselines than silica gel layers.

In order to obtain reasonable reproducibility, the scanning must be made with care. Important influencing factors, some of which have been investigated previously[1,8,17] are: (a) positioning of the scanner; (b) standardisation of time between end of chromatography and start of scanning; (c) loading volume; (d) developing distance; (e) layer thickness. The first two (a and b) have been found very important

TABLE II

AREAS OF FLUORESCENCE PEAKS OF DANS-AMINO ACIDS (2 μg EACH) ON POLYAMIDE AND SILICA GEL LAYERS (*cf.* TEXT AND TABLE I)

DANS-	*Polyamide*		*Silica Gel*	
	Peak area[a] (mm^2)	t[b] (*min*)	*Peak area*[a] (mm^2)	t[b] (*min*)
Glutamic acid	2230	1	1500	6
Di-cystine	1870	1.5	2000	1.5
Methionine[c]	3860	10.5	2110	13
Tryptophane[c]	1850	13	740	9.5
Glycine	3530	19.5	2050	15
Serine[c]	2510	20.5	1460	22.5
Leucine[d]	3430	24.5	2010	23.5
Alanine[c]	4060	26.5	1910	28.5
Phenylalanine	3620	32.5	2300	38.5
Threonine[c]	3380	32.5	1520	35.5
Aspartic acid[d]	1570	35.5	960	32
Proline[c]	3600	44.5	1840	46.5
Hydroxyproline[d]	1590	49.5	1330	47
Valine[d]	4020	55	2190	57
Isoleucine[d]	4090	69.5	2290	68.5
Asparagine[d]	1890	79	1630	77.5

[a] Planimetry (5 times).
[b] Time (min) between plate drying and scanning (see METHODS).
[c] Cyclohexylamine salt.
[d] Piperidine salt.

TABLE III

AREAS OF FLUORESCENCE QUENCHING PEAKS OF DNP-AMINO ACIDS (2 μg EACH) ON POLYAMIDE AND SILICA GEL LAYERS (*cf.* TEXT AND TABLES I AND II)

DNP-	*Polyamide*		*Silica gel*	
	Peak area (mm^2)	*t* (*min*)	*Peak area* (mm^2)	*t* (*min*)
Di-Lysine	3160	0	2480	1
Cystine	2260	1	1460	1
Taurine	540	1	2190	4
Glycine	2800	1	1560	2.5
Cysteine	4340	2.5	2440	5
Glutamic acid	2150	3	1840	4.5
Arginine	3060	3	1020	1.5
Aspartic acid	2270	4	1020	7.5
Di-Homocystine	1610	12.5	1410	11.5
Alanine	3720	13	3290	20.5
Di-Cystine	1420	13.5	1160	15
Di-Homocysteine	3170	17.5	2100	14
Hydroxyproline	2070	17.5	1850	11
Leucine	4870	18	2570	21.5
Glutamine	1830	22	800	25.5
ε-Lysine	3540	24	3890	20
Methioninsulfone	3800	25.5	2100	24.5
Methioninsulfoxide	2220	26	1000	31.5
Isoleucine	4660	26	2210	29.5
Citrulline	4090	27	1690	14.5
β-Alanine	3990	29	2920	26
Di-Histidine[a]	2640	31.5	690	37
Phenylalanine	3260	34	2540	36
Homocitrulline	3840	35	1950	33
γ-Amino butyric acid	3780	38	1750	35.5
Tryptophane	2930	38.5	1850	41
Di-Tyrosine	2580	38.5	1910	35
α-Amino isobutyric acid	3640	40	2240	42
Proline	3930	44.5	3390	44.5
Sarcosine	5550	45	3510	50.5
Threonine	3050	46.5	1110	45.5
Serine	1750	49	1300	58
Hydroxylysine	1470	50.5	900	53
α-Amino butyric acid	4750	51.5	2710	58
Valine	3190	53	1990	59.5
Cysteic acid	3290	59.5	1760	51
im-Histidine	3010	60	2200	65
α-Amino adipic acid	3030	63	1770	78
Asparagine	1300	63	420	64
Methionine	3060	64.5	1530	66
Mono-O-Tyrosine	1880	65	420	81

[a] Impure, gives two spots on polyamide layers.

with respect to obtaining accurate results[8,15,17]. SEILER[7] reported on the influence of the moisture content of the layer on fluorescence. This effect, which has also been confirmed by us[8], can be suppressed by spraying the chromatogram with triethanolamine–isopropanol (1:4)[7,15]. Moreover the spray-technique of SEILER[7] increases the intensity of fluorescence. In addition, it has been noticed that heating the chromatograms prior to scanning in many cases caused a considerable increase in the amount of fluorescence[21,22]. The extent to which fluorescence quenching peaks are influenced

TABLE IV

AREAS OF FLUORESCENCE QUENCHING PEAKS OF PTH-AMINO ACIDS (2 μg) ON POLYAMIDE AND SILICA GEL ZINC SILICATE LAYERS (*cf.* TEXT AND TABLES I AND II)

PTH-	*Polyamide*		*Silica gel*	
	Peak area (mm^2)	*t* (*min*)	*Peak area* (mm^2)	*t* (*min*)
Δ-Serine	560	1	440	7
α-Amino butyric acid	1710	1.5	2310	1.5
Cystine	750	2	750	8
Alanine	2230	3	2300	2
Histidine			740	3
Glutamic acid	2060	9	1380	16
Homocitrulline	1520	11.5	1130	16.5
Leucine	1950	12.5	2540	16
Glycine	2120	16	1820	20
Valine	2670	22.5	2250	19
Phenylalanine	2490	27.5	1270	30.5
Methionine	2630	36.5	1800	34
Proline	2220	40	2660	40.5
Citrulline	1180	40	1150	44
Glutamine	2180	49	2160	52
Tryptophane	970	60.5	1570	60.5
Hyproline	1590	65.5	1390	62
α-Amino isobutyric acid	1320	84.5	1310	84
Methioninesulfone	2260	91.5	1240	78
Cysteic acid	1020	112.5	1210	105.5
Arginine	3140	124.5	1190	123
Asparagine[a]	1430	137.5	850	140
Aspartic acid	1710	142	1780	132
Lysine	1620	156	1560	147
Tyrosine	1340	171	1490	158
Isoleucine	1730	180	1300	168
Δ-Threonine	820	191	1250	187.5
Threonine	1270	197	1780	181.5

[a] Impure, gives a secondary spot.

by the time between drying and scanning has also been investigated[1,8,15]. Since the quenching peaks of some U.V.-absorbing substances have also shown time-dependence[1,8,15], it is a prerequisite in all cases of *in situ* scanning-fluorescence as well as quenching—to check whether the influence of time on the results has to be taken into account. The remaining influencing factors were found to be of less importance, however, their standardisation is also advisable.

Different techniques for the measurement of peak areas have been investigated. In the case of symmetrical peaks, with a uniform baseline, simple geometric measurement, planimetry and the use of a disc integrator have about the same accuracy. However, if the peaks are nonsymmetrical, which often occurs in the case of fluorescence quenching, planimetry gave the best reproducibility (Table V). The relative standard deviations* amount to 3.5–5 % in the case of fluorescence, and 5–7 % in the case of fluorescence quenching (measurement of 6 spots on the same chromatogram by planimetry). If spots from different chromatograms are to be compared,

* In all experiments the "*t*-values" (see *e.g.* Fig. 3) were kept constant. The "spray-technique" of SEILER[7] has been found less reproducible, particularly if spots from one chromatogram to another had to be compared[15] (*cf.* also ref. 23).

TABLE V

REPRODUCIBILITY OF FLUORESCENCE QUENCHING USING DIFFERENT MEASUREMENT TECHNIQUES (VALUES ON SIX DIFFERENT CHROMATOGRAMS: DNP-PROLINE, 2 μg)

Chromatogram	*Peak area* (mm^2)		
	Planimetry[a]	*Geometry*[b]	*Integrator units*[c]
1	2460	3105	870
2	2350	2625	730
3	2570	3055	790
4	2650	3420	900
5	2250	2635	745
6	2100	2510	665
Mean value	2400	2890	785
Relative standard deviation	8.6%	12.3%	11.3%

[a] Measurement 5 times.
[b] Height·width at half-height.
[c] A disc integrator is used. The baseline has to be adjusted to the *same* value, before measurement.

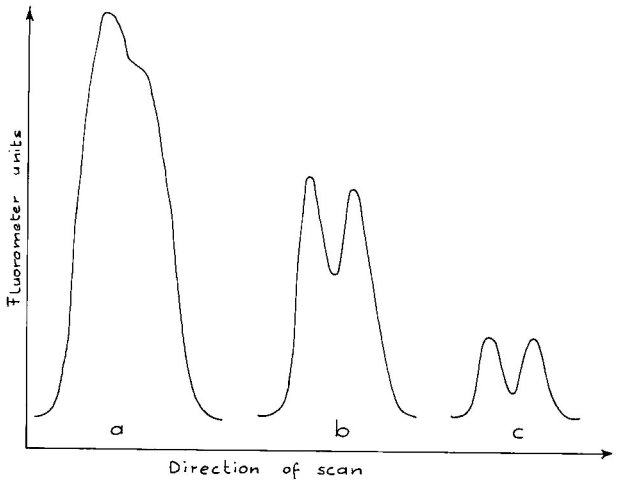

Fig. 6. Resolution of peaks of two DANS-derivatives (2 μg each). Measuring of fluorescence according to the conditions given in Table I; (a) aperture to door 3.4 mm; (b) aperture to door 1.7 mm; (c) aperture to door 0.85 mm.

relative standard deviations of about 10–15% can be obtained. An example is shown in Table V. Finally, the resolution of closely adjacent spots has been investigated. Fig. 6 shows an experiment where there is almost no resolution of two fluorescence peaks of DANS-derivatives, using the standard procedure as given in Table I. If, however, the aperture of the door is adjusted to only $^1/_2$ or even $^1/_4$ (*i.e.* 1.7 and 0.85 mm) the resolution of peaks increased considerably. The sensitivity, which is of course lower in this case, can be increased as much as is convenient by the use of other neutral density filters[23]. Nevertheless, the scanning of closely adjacent spots is sometimes difficult. The scanning of spots on two-dimensional chromatograms is troublesome and not free from error. This problem is now being investigated.

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CHROM. 3694

RAPID FLUORIMETRIC METHODS BY INSTANT THIN-LAYER CHROMATOGRAPHY FOR THE ASSAY OF PHENALEN-1-ONE AND 7H-BENZ(*de*)-ANTHRACEN-7-ONE IN POLLUTED URBAN ATMOSPHERES

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SUMMARY

Through the use of glass-fiber paper impregnated with silica gel (called instant thin-layer chromatography, or ITLC), the rapid determination of phenalen-1-one (PO) and 7H-benz(*de*)anthracen-7-one (BO) is facilitated. One method is introduced for the specific determination of PO, and one method for the determination of both PO and BO at the same time. Both methods are superior to previous methods in terms of speed, selectivity, simplicity, and sensitivity. In addition, they give reasonable accuracy and precision.

Samples from urban atmospheres and air pollution source effluents can be analyzed rapidly for PO and BO by these methods. Analysis of one sample of organic extracts would take less than 30 minutes; analysis of a dozen samples would take less than 2 h. For those laboratories without instrumentation the amount of PO and BO can be estimated by eye after separation; eight analyses for either compound can be accomplished in about 20 minutes.

INTRODUCTION

The first indication of the presence of phenalen-1-one (PO) and the carcinogenic 7H-benz(*de*)anthracen-7-one (BO) in polluted air was obtained by SAWICKI AND JOHNSON[1]. At that time, the use of trifluoroacetic acid fumes (TFA) for locating basic compounds on a thin-layer chromatogram was introduced. The chromatograms from urban air samples separated on aluminum oxide with pentane–ether (19:1, v/v) as the developer, were found to contain, after TFA fuming, a brilliant emerald green spot and a moderately brilliant pink-to-orange fluorescent spot. Because these fluorescent colors faded rather rapidly but could be brought back by retreatment with TFA fumes, it was believed that these two compounds were weakly basic, and probably aromatic carbonyl compounds.

Examination of the column and thin-layer chromatographic separation and the fluorescence characteristics of a large number of polynuclear ring carbonyl compounds[2]

disclosed that two of the compounds, PO and BO, showed separation and fluorescence color properties similar to those of the two unknown spots.

Through the use of thin-layer chromatography and direct spectrophotofluorimetric examination of thin-layer plates[3], the emerald green and orange TFA-treated spots were identified as PO and BO, respectively, and their amounts in airborne particulate samples were estimated.

Based on the previous work a group of eight methods involving TLC separation, filter fluorometry, quenchofluorimetry, and spectrophotofluorimetry were developed and compared[4].

In the work described in this paper, use was made of fast separation on glass-fiber paper impregnated with silica gel (ITLC) to develop methods for the determination of phenalen-1-one and 7H-benz(*de*)anthracen-7-one that are faster, simpler and as accurate as the previously described methods.

REAGENTS AND APPARATUS*

Phenalen-1-one (perinaphthenone) and 7H-benz(*de*)anthracen-7-one (benzanthrone) were obtained from the Aldrich Chemical Company, Milwaukee, Wisc., and crystallized to a constant melting point. The remainder of the reagents were obtained from commercial sources and crystallized or distilled until they were chromatographically pure.

Chromatography was performed on glass-fiber paper impregnated with silica gel. This type of separation is called instant thin-layer chromatography (ITLC) by the Gelman Instrument Company, Ann Arbor, Mich. The chromatograms were examined for fluorescence colors in a Chromato-Vue cabinet (Kensington Scientific Corp., 1717 Fifth Street, Berkeley 10, Calif.) under a 3660 Å light source.

An Aminco-Bowman spectrophotofluorimeter was used in determining fluorescence spectra in solution, with the following settings: sensitivity 50, slit arrangement No. 2, and phototube RCA type 1P21. In the assay of PO, Corning filter No. CS3-72 (Corning Glass Works, Corning, N.Y.) was used to exclude wavelengths below 435 mμ. Where indicated in the procedure, Aminco filter No. 4-7164 (American Instrument Company, Silver Springs, Md.) was used in the assay of BO to exclude wavelengths below 535 mμ.

Procedure for assay of phenalen-1-one

The separation of extracts of urban airborne particulates and the subsequent location of PO in the samples were performed with pentane–trifluoroacetic acid (50:1, v/v) as the developer; 20 minutes were required for the solvent front to travel 15 cm. After development, the emerald green spot was located under ultraviolet light and intensified with the use of TFA fumes. The spot was then cut out and extracted with three 1-ml volumes of boiling acetone; the resulting extract was evaporated to dryness in a boiling water bath. The residue was dissolved in 0.5 ml of concentrated sulphuric acid, and a reading was then obtained at an emission wavelength of 498 mμ with the spectrophotofluorimeter set at an excitation wavelength of 400 mμ. Filter No. CS3-72 was used in the instrument to exclude wavelengths below 435 mμ. In this

* Mention of commercial products does not constitute endorsement by the Public Health Service.

fashion much of the background fluorescence that interfered with the analysis of air samples was removed. The standard PO spot and a spot used as the blank, of the same size and R_F value as the unknown and standard spots, were cut out and treated in the same manner as the unknown.

Investigation of various temperatures and volumes of acetone in the extraction procedure proved that extraction with three successive 1-ml volumes of boiling acetone was sufficient for quantitative extraction.

Calculation of the amount of PO in the unknown sample was based on the linear relation of fluorescence intensity *versus* concentration. Conformance with Beer's law was observed from 20 to at least 1000 ng.

Procedure for assay of phenalen-1-one and 7H-benz(de)anthracen-7-one

The location of both PO and BO in the extracts of urban airborne particulates was accomplished with pentane–methylene chloride (3:1, v/v) as the developer. About 18 min were required for the solvent front to travel 15 cm. A separation time of 6 min was possible when the solvent front was allowed to travel 10 cm. After development, the paper was examined under ultraviolet light, and the PO and BO spots, opposite the appropriate standards, were located with TFA fumes. The extraction technique and estimation of PO in the sample were done as described in the preceding section. Estimation of BO was made at an excitation wavelength of 370 mμ with the spectrophotofluorimeter set at an emission wavelength of 560 mμ. Calculation of the amount of BO in the unknown sample was based on the linear relation between the fluorescence intensity of BO and its concentration. Conformance with Beer's law was observed from 10 to at least 1000 ng. For this calculation, the amount of transmittance between the wavelength maximum of 370 mμ and the wavelength minimum of 390 mμ was read. A Beer's law relationship existed if the readings were made with the instrument set at maximum sensitivity of 50 or at a sensitivity of 25.

An alternative reading procedure was necessary for one sample that was difficult to analyze by the method described because of the unusually low amounts of BO present. With Aminco filter No. 4-7164, the reading was taken at emission wavelength 560 mμ with the instrument set at an excitation wavelength of 360 mμ.

Estimation of PO and BO by eye

The unknown sample and spots containing PO and BO in 0.025, 0.05, 0.1, 0.2, and 0.4 μg amounts were separated by the pentane–methylene chloride procedure. The chromatogram was placed under ultraviolet light. All spots were sprayed with trifluoroacetic acid fumes and the amount of PO and BO in the unknown sample was estimated immediately by eye through comparison of the unknown and standard spots. The PO method could also be used.

RESULTS AND DISCUSSION

The method for the determination of PO with pentane–trifluoroacetic acid (50:1, v/v) as the developer has the advantage that the analysis can be performed in much less time than by previous literature methods, with the result that more samples can be analyzed in the same amount of time. In addition, the method is simple, sensitive, and selective, and the aza heterocyclic compounds do not interfere because they

remain at the origin. Fig. 1 shows the emerald green spot found in the separation of 1 mg of coal-tar-pitch and 1 mg of a benzene-soluble fraction of urban airborne particulate, which gives the same fluorescence color and R_F value as the PO standard. Both the BO and 9-xanthenone standards traveled to the solvent front. The mixture of aza heterocyclic compounds that remained at the origin includes 1 μg each of benzo(*f*)quinoline, benzo(*h*)quinoline, benz(*a*)acridine, and benz(*c*)acridine.

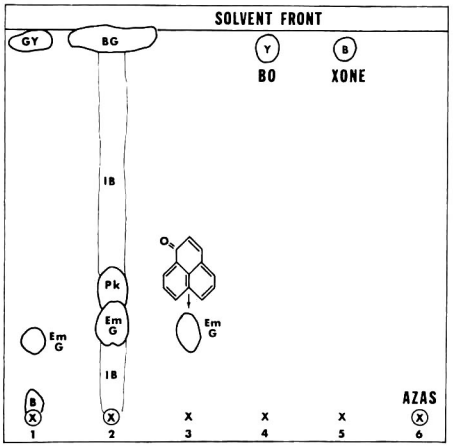

Fig. 1. Thin-layer chromatogram on silica gel glass-fiber paper with pentane–trifluoroacetic acid (50:1, v/v) as the developer. The fluorescence colors of the compounds shown appeared after the chromatogram had been sprayed with trifluoroacetic acid fumes. Color abbreviations are as follows: B = blue, Em G = emerald green, GY = green yellow, O = orange, BG = blue green, Pk = pink, Y = yellow, l = light. Amounts of the compounds separated are as follows: (1) 1 mg of coal-tar-pitch, (2) 1 mg of a benzene-soluble fraction of urban airborne particulates, (3) 1 μg of phenalen-1-one, (4) 1 μg of 7H-benz(*de*)anthracen-7-one, (5) 1 μg of 9-xanthenone, and (6) a mixture containing 1 μg each of benzo(*f*)quinoline, benzo(*h*)quinoline, benz(*a*)acridine, and benz(*c*)acridine.

The method for the determination of BO and PO with pentane–methylene chloride (3:1, v/v) as the developer offers the advantage that a large number of samples can be assayed for these two compounds in a relatively short time because separation time is reduced to 18 min or less, depending on the distance the solvent front is allowed to travel. The results calculated for PO by this procedure are analogous to those obtained by the specific procedure for PO.

Since PO and BO can be determined at the 10 to 20 ng level both methods provide a high order of sensitivity. Compared to all previous procedures, both methods are superior in simplicity and speed. The methods show high selectivity, since we knwh of no interferences in the determination of PO and BO by these methods. Althougo the natural mixtures contain many thousands of compounds, the PO and BO spots are so easily obtained and stand out so vividly on the chromatograms that the procedure should prove useful for teaching purposes.

Because of the vividness of the spots, PO and BO can be estimated by eye with the help of standards. The spots must be fumed with trifluoroacetic acid and read immediately. Although the emerald green color of the PO spot fades gradually, it is still vivid after several hours. PO must be more basic than BO, since the orange color

of the BO cation fades more quickly. Since this fading or loss of trifluoroacetic acid is much more rapid on paper chromatograms or on alumina or silica gel thin-layer plates, the silica gel glass-fiber paper must hold trifluoroacetic acid much more tenaciously. This phenomenon should prove of value in future research.

Recoveries of PO and BO from an extract of urban airborne particulates collected indoors were studied. Under the conditions of the analysis the material gave an indication of less than 10 μg of both PO and BO per gram of particulate. The two chemicals were added to this particulate and a Soxhlet extraction for 6 h was performed with benzene in one flask and methylene chloride in another flask. These extracts served as the "test" extracts. At the same time, a 6-h Soxhlet extraction of the particulate without the added BO and PO was carried out, and these extracts represented "blanks" in the analyses. After analysis in triplicate for PO and BO by the respective methods, the results were as follows: PO was recovered from the benzene extract in a 101 % yield and from the methylene chloride extract in a 104 % yield; BO was recovered from the benzene extract in a 91 % yield and from the methylene chloride extract in a 106 % yield. Because of the small amount of BO and the presence of unknown interfering substances in this particular sample, the final step in the assay of the extract for BO was modified slightly. Instead of obtaining a reading at the excitation wavelength of 370 mμ with the spectrophotofluorimeter set at an emission wavelength of 560 mμ, the reading was taken at the emission wavelength of 560 mμ with the instrument set at an excitation wavelength of 360 mμ. An Aminco filter No. 4-7164 was also used; this filter, which excluded light of wavelengths less than 535 mμ, proved to be the best one for maximum sensitivity and minimum interference from other compounds. The modification appeared to work well in this analysis and could be used in place of the original technique when the amount of BO in the sample is low.

A study involving the efficiency of extraction of the PO and BO spots from the silica gel glass-fiber paper was also conducted. Results obtained for BO were slightly higher when chloroform was used to extract the paper than when acetone was used; the results for PO, however, were the same with chloroform and acetone. In all cases the efficiency of extraction from the paper for both PO and BO was close to quantitative, regardless of which solvent was used.

APPLICATION

The separation of a 1 mg sample of a benzene-soluble fraction of urban airborne particulates from Greenville, S.C., and the subsequent identification of BO and PO in the sample are shown in Fig. 2. The fluorescence spectra in Fig. 2 are those of the extract from the standard BO spot and the extract from the unknown spot opposite the BO standard.

The assay results for PO and BO in the benzene-soluble fraction of urban airborne particulates are shown in Table I.

For the estimation of PO in the samples, the specific method for PO was used, with pentane–trifluoroacetic acid (50:1, v/v) as the developer. Results obtained with and without a filter in the instrument were compared. The average value clearly shows that the use of the filter reduced the percent deviation considerably. In addition, the value thus obtained for PO was comparable to that found by an earlier method[3].

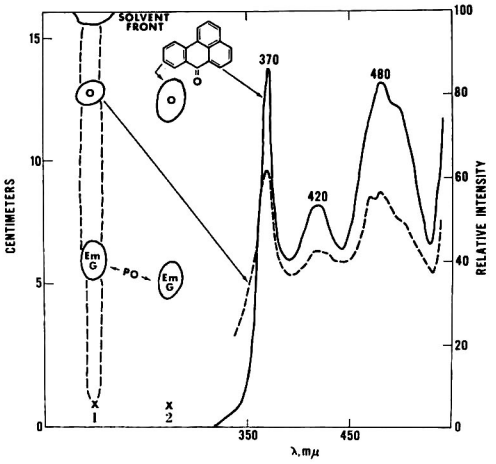

Fig. 2. On the left side appears the separation on silica gel glass-fiber paper of (1) 1 mg of a benzene-soluble fraction of urban airborne particulate from Greenville, S.C., and (2) a mixture containing 0.8 μg of phenalen-1-one and 0.8 μg 7H-benz(*de*)anthracen-7-one, with pentane–methylene chloride (3:1, v/v) as the developer. The fluorescence colors shown appeared after TFA fuming. For color abbreviations refer to Fig. 1. On the right side are shown the excitation spectra at emission λ 560 and meter multiplier (MM) 0.01 of (———) the extract from the BO standard spot in 0.5 ml concentrated sulfuric acid and (– – –) the extract from the unknown spot opposite the BO standard spot in 0.5 ml concentrated sulfuric acid.

TABLE I

ASSAY FOR PHENALEN-1-ONE AND 7H-BENZ(*de*)ANTHRACEN-7-ONE IN THE BENZENE-SOLUBLE FRACTION OF URBAN AIRBORNE PARTICULATES[a]

Sample size (mg)	*PO method[b] (mg/g benzene-soluble fraction)*	*Sample size (mg)*	*PO method[c] (mg/g benzene-soluble fraction)*	*Sample size (mg)*	*BO method (mg/g benzene-soluble fraction)*
1.0	0.33	0.6	0.25	1.0	0.29
1.0	0.27	0.6	0.27	1.0	0.30
1.0	0.24	0.6	0.24	1.0	0.35
1.0	0.24	0.6	0.25	1.0	0.33
0.8	0.28	0.6	0.29	1.0	0.27
0.6	0.20	0.6	0.29	1.0	0.53
0.4	0.23	0.6	0.27	1.0	0.46
0.04	0.33	0.6	0.24	1.0	0.35
		0.4	0.26		
Average[d]	0.26 ± 0.047		0.26 ± 0.019		0.36 ± 0.12

[a] Corrected values based on recovery data.
[b] Determination without filter.
[c] Determination with filter No. CS3-72.
[d] Average value of 0.2 mg PO/g was obtained by previous method[3].

Investigation of various filters for use in the instrument for the assay of PO showed that Filter CS3-72 was superior to the other filters. A 0.6 mg sample of a benzene-soluble fraction of urban airborne particulate from New Rochelle, N.Y., was separated and analyzed for PO in the prescribed manner. Fig. 3 shows the spectra of

the extract from the unknown spot opposite the PO standard, run without a filter and with various filters placed in the instrument. This investigation indicated that filter No. CS3-72 should be used in the assay for PO to give maximum sensitivity and minimum interference from other fluorescing compounds in the mixture present in the spot.

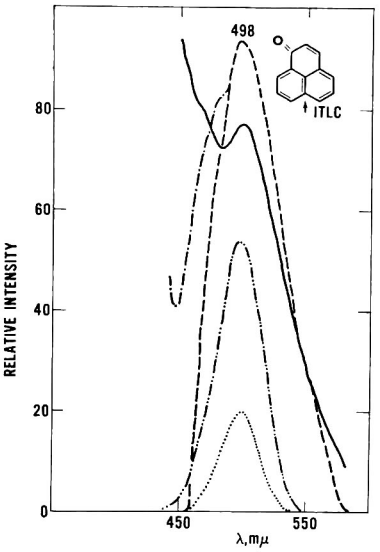

Fig. 3. Effect of various filters on the emission spectrum of the extract of an unknown spot opposite the phenalen-1-one spot from the separation of 0.6 mg of a benzene-soluble fraction of urban airborne particulate from New Rochelle, N.Y., with pentane–trifluoroacetic acid (50:1, v/v) as the developer. The readings were taken at an emission λ of 498 mμ with the excitation λ set at 400 mμ. The extract was in 0.5 ml of concentrated sulfuric acid. (———) No filter, MM = 0.01. (– – –) Corning Glass filter No. CS3-72, MM = 0.01; cuts off light below 435 mμ. (–·–·) Corning Glass filter No. CS3-70, MM = 0.01; cuts off light below 490 mμ. (–· ·–· ·) Filter No. 65A, MM = 0.01, Aminco Catalogue No. 4-7160; peaks at 495 mμ. (·····) Filter No. 75, MM = 0.01, Aminco Catalogue No. 4-7120; peaks at 490 mμ.

Table I also gives the average value of BO found in 1 g of the benzene-soluble fraction of urban airborne particulates. The assay was performed according to the described procedure, with pentane–methylene chloride (3:1, v/v) as the developer.

All the values listed for PO and BO in the tables represent corrected values based on recovery data, the results of which will be discussed in a later section.

The results of the assay for BO and PO by their specific methods, in samples from the urban atmospheres of various cities are shown in Tables II and III. An appropriate amount ($\sim$ 1 mg) of the urban sample to be analyzed was spotted on a silica gel glass-fiber paper, and the analysis for either BO or PO was performed according to the described methods.

The values for BO and PO listed for the various cities represent a general means of comparison. The values do not represent the specific amounts present in each city, for this would involve the analysis of numerous samples collected in many parts of each city and at different times of the year. If this were done, an overall average value of each city could be calculated, and the values for the cities specifically compared.

TABLE II

ASSAY[a] FOR 7H-BENZ(*de*)ANTHRACEN-7-ONE ON ITLC

Location[b]	*mg BO/g sample*[c]	*Average value*
Harrisburg, Pa.	0.13, 0.20, 0.10	0.14
Greenville, S.C.	0.40, 0.43, 0.48	0.44
Ashville, N.C.	0.46, 0.45, 0.50	0.47
Mt. Vernon, N.Y.	0.22, 0.22, 0.22	0.22
Lynn, Mass.	0.20, 0.25, 0.28	0.24
New Rochelle, N.Y.	0.13, 0.14, 0.16	0.14
Memphis, Tenn.	0.20, 0.23, 0.25	0.23
Ft. Wayne, Ind.	0.23, 0.31, 0.29	0.28
Wilkes-Barre, Pa.	0.13, 0.14, 0.14	0.14
Glen Cove, N.Y.	0.14, 0.13, 0.13	0.13
Saginaw, Mich.	0.25, 0.34, 0.32	0.31

[a] Developer: pentane–methylene chloride (3:1, v/v); adsorbent: glass-fiber paper impregnated with silica gel; time for solvent front to travel 15 cm = 18 min.
[b] Sample size was 1 mg for each determination.
[c] Corrected value based on recovery data.

TABLE III

ASSAY[a] FOR PHENALEN-1-ONE ON ITLC

Location[b]	*mg PO/g sample*	*Average value*
Memphis, Tenn.	0.22, 0.17, 0.17	0.19
Saginaw, Mich.	0.33, 0.30, 0.33	0.32
Harrisburg, Pa.	0.22, 0.21, 0.22	0.22
Glen Cove, N.Y.	0.12, 0.09, 0.16	0.12
Wilkes-Barre, Pa.	0.13, 0.12, 0.14	0.13
Ft. Wayne, Ind.	0.43, 0.41, 0.43	0.42
New Rochelle, N.Y.	0.24, 0.25, 0.22	0.24
Mt. Vernon, N.Y.	0.12, 0.11, 0.12	0.12
Lynn, Mass.	0.23, 0.25, 0.23	0.24
Ashville, N.C.	0.52, 0.52, 0.51	0.52
Greenville, S.C.	0.38, 0.36, 0.40	0.38

[a] Developer: pentane–TFA (50:1, v/v); adsorbent: glass-fiber paper impregnated with silica gel; time for solvent front to travel 15 cm = 20 min; filter No. CS3-72 used in SPF.
[b] Sample size in all determinations was 0.6 mg except for Ashville, N.C. and Greenville, S.C., which were 0.4 mg samples.

Nevertheless, the values listed in these tables do give a general idea as to the differences in amounts of BO and PO found in the cities.

Fig. 4 shows the emission spectrum of the extract of the unknown emerald green spot opposite the PO standard spot obtained in the separation of 0.4 mg of a benzene-soluble fraction of urban airborne particulate from Ashville, N.C. The spectrum for the unknown is the average of triplicate determinations made on this sample. The emission spectrum of the PO standard is shown, as well as that of the blank, which was cut out at the same R_F value as that of the standard and unknown. All the spots were extracted in the same way.

In an attempt to ascertain the presence of BO and PO in samples from air pollution sources, an extract of an effluent from a coffee-roasting industrial plant was

separated. The results indicated the presence of two well-separated spots of BO and PO, which gave the same R_F values as their respective BO and PO standards and the same fluorescent colors with TFA fumes. This preliminary work indicates the presence of these two compounds in various types of polluted atmospheres. The procedures should be used in the future investigation of various samples of polluted atmospheres for BO and PO.

For those laboratories without instrumentation, the amounts of PO and BO can be estimated by eye after separation. This means that eight complete analyses can

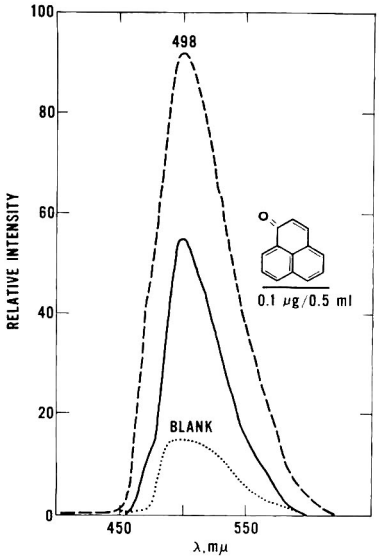

Fig. 4. Emission spectra of extracts from silica gel glass-fiber paper developed with pentane–trifluoroacetic acid (50:1, v/v) and read at F 400/498. All extracts were read in 0.5 ml concentrated sulfuric acid. (– – –) Unknown spot opposite phenalen-1-one standard in the separation of 0.4 mg of a benzene-soluble fraction of urban airborne particulate from Ashville, N.C. Average of three determinations, MM·T = 0.81 ± 0.01. (———) 0.1 µg of phenalen-1-one. (·····) Blank, scan area cut out at same R_F value as standard and unknown. All spots were extracted in the same manner.

TABLE IV

ESTIMATION OF PHENALEN-1-ONE AND 7H-BENZ(*de*)ANTHRACEN-7-ONE BY EYE

Sample site	*PO*[a] (*mg/g benzene-soluble fraction*)		*BO*[b] (*mg/g benzene-soluble fraction*)	
	SPF	*Eye*	*SPF*	*Eye*
Saginaw, Mich.	0.29	0.30	0.28	0.25
Harrisburg, Pa.	0.20	0.15	0.13	0.20
Ft. Wayne, Ind.	0.39	0.30	0.25	0.15
New Rochelle, N.Y.	0.22	0.15	0.13	0.10
Lynn, Mass.	0.22	0.20	0.22	0.20

[a] Sample size for analysis: 0.6 mg of benzene-soluble fraction.
[b] Sample size for analysis: 1.0 mg of benzene-soluble fraction.

be done for either compound in about 20 min; the only equipment required is glass-fiber paper impregnated with silica gel, a developing tank, an ultraviolet light, pentane, trifluoroacetic acid, methylene chloride, phenalen-1-one, 7H-benz(*de*)anthracen-7-one, and reasonably good eyesight. The results obtained by this estimation are compared with those given by the spectrophotofluorimetric method in Table IV.

CONCLUSIONS

With the described separation methods and the use of glass-fiber paper impregnated with silica gel, the determination of phenalen-1-one and the carcinogen, 7H-benz(*de*)anthracen-7-one, can be performed in a fraction of the time necessary for previous methods. In addition, these methods are simple, selective, and sensitive. Preliminary investigation shows that urban atmospheres and air pollution source effluents can be analyzed for the presence of PO and BO by these methods. For those laboratories without fluorescence instrumentation PO and BO can be readily estimated by eye under ultraviolet light after separation of the air sample and appropriate standards on silica gel glass-fiber paper.

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CHROM. 3707

TRENNUNG VON BASEN, NUCLEOSIDEN UND NUCLEOTIDEN AUF KATIONENAUSTAUSCHERN*,**

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SUMMARY

The separation of bases, nucleosides and nucleotides on cation exchangers

The separation and determination of bases and nucleosides on cation exchangers using ammonium formate buffers were studied systematically and the results have been summarised in diagrams. Further possibilities of separation with water and diluted HCl are described. All methods were used in routine work. The separation of nucleotides (only pure substances) on cation exchangers is also described.

COHN hat 1949 die wesentlichen Bedingungen für die Trennungen der einzelnen Nucleinsäurederivate auf Kationen- und Anionenaustauschern beschrieben[1,2]. In zahlreichen späteren Arbeiten wurden die Angaben von COHN modifiziert und erweitert. Ausführliche Übersichten finden sich bei SAUKKONEN[3] und COHN[4–6].

Die Bestimmung der Basen und Nucleoside auf Kationenaustauschern erfolgte bisher fast immer nach den Angaben von COHN mit 0.25 bis 2 *N* HCl. Benutzt man jedoch Puffer in pH-Bereichen von 2–4, lassen sich zahlreiche Basen und Nucleoside

* Auszugsweise vorgetragen auf der Nordwestdeutschen Chemiedozententagung in Clausthal-Zellerfeld 1965[13] und teilweise in der Habilitationsschrift, Hamburg, 1966, veröffentlicht[9].

** Abkürzungen:

Ad	= Adenin	IMP	= Inosinmonophosphat
Ado	= Adenosin	Ino	= Inosin
AMP	= Adenosinmonophosphat	1MeAd	= 1-Methyl-adenin
2′-, 3′- und 5′-AMP	= 2′-, 3′- und 5′-Adenosinmonophosphat	6MeAd	= 6-Methyl-adenin
		6diMeAd	= 6-Dimethyl-adenin
dAMP	= Deoxyadenosinmonophosphat	5MeCy	= 5-Methyl-cytosin
		NAD^+	= Nicotinamid-adenin-dinucleotid
CMP	= Cytidinmonophosphat		
dCMP	= Deoxycytidinmonophosphat	$NADP^+$	= Nicotinamid-adenin-dinucleotidphosphat
Cy	= Cytosin		
Cyd	= Cytidin	Nid	= Nicotinsäureamid
dCyd	= Deoxycytidin	Th	= Thymin
GMP	= Guanosinmonophosphat	Thd	Thymidin
dGMP	= Deoxyguanosinmonophosphat	dThd	= Deoxythymidin
Gu	= Guanin	ThMP	= Thymidinmonophosphat
Guo	= Guanosin	UMP	= Uridinmonophosphat
Ha	= Harnsäure	Urd	= Uridin
Hy	= Hypoxanthin	Xa	= Xanthin

aufgrund der verschiedenen pK-Werte der Aminogruppen trennen, die auch für die elektrophoretische Trennung verantwortlich sind. CRAMPTON *et al.*[7] haben eine Trennung von Basen mit einem Citratpuffer beschrieben. Wir haben die Retentionswerte von Purin- und Pyrimidinderivaten in den oben genannten pH-Bereichen und bei verschiedenen Salzkonzentrationen zusammengestellt. So kann man für eine gewünschte Trennung einen günstigen pH-Wert und die richtige Salzkonzentration des Puffers heraussuchen.

Daneben lassen sich Basen und Nucleoside ohne Aminogruppen sowie Nucleotide durch Adsorption an die Matrix des Austauschers trennen. Austausch- und Adsorptionseffekte überlagern sich in einem Ausmass, dass man für Purinverbindungen nur mit Einschränkungen von Ionenaustauschchromatographie sprechen kann. Die Trennungen wurden für Untersuchungen des Abbaues der freien Nucleotide in Geweben[8–11] und für Arbeiten über den Erythrocytenstoffwechsel eingesetzt[12].

METHODE

Wir verwendeten die Austauscher Dowex* bzw. AG** 50W X4, 200–400 mesh oder —400 mesh. Die teuren sphärischen Harze mit einheitlicher Korngrösse wurden wegen des hohen Preises nicht benutzt, obwohl sie wesentlich bessere Trennungen versprechen. Schon die intensiver nachbehandelten AG-Harze vergrössern die Trennleistung. Die Säule hatte einen Durchmesser von 0.9 cm und das Harz wurde 15–20 cm hoch gepackt. Bei den Versuchen unter optimalen Bedingungen eine grösstmögliche Anzahl von Substanzen zu trennen, arbeiteten wir mit 50 cm langen Säulen. Mit einem Fraktionssammler*** und einem 10 ml Syphon wurden die einzelnen Fraktionen quantitativ aufgefangen und mit einem Durchflussphotometer*** sowie einem Zweifarbenschreiber*** markiert. Sie konnten dann zusammengeschüttet und im Spektralphotometer gemessen werden. Eichwerte publizierten wir schon früher[14] und die molaren Extinktionskoeffizienten wurden der Literatur entnommen[15,16]. Die Durchflussgeschwindigkeit betrug 36 cm/h, der Durchfluss erfolgte durch Überdruck (0.2–0.5 atü Stickstoff) oder mit Hilfe einer Schlauchpumpe§. Wir verwendeten Ammoniumformiatpuffer, die gegenüber den Citratpuffern den Vorzug der geringeren U.V.-Absorption und der grösseren Flüchtigkeit besitzen, falls Eluate zur Rechromatographierung einmal eingeengt werden müssen. Für den pH-Bereich von 2.5–4 wurde eine 0.14 *N* HCOOH mit NH_3-Lösung (25 %, $D = 0.91$) auf den entsprechenden pH-Wert elektrometrisch eingestellt. Der pH-Wert von 2 wurde durch vermehrte Zugabe von HCOOH erreicht. Der Natriumformiatpuffer im zweiten Abschnitt wurde hergestellt, indem wir eine 0.05 *N* NaOH Lösung mit konzentrierter Ameisensäure auf die entsprechenden pH-Werte titrierten.

ERGEBNISSE UND DISKUSSION

Zuerst sollen die Trennungen mit Reinsubstanzen beschrieben und daran anschliessend soll über einige Anwendungen mit biologischem Material berichtet werden.

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* The Dow Chemical Company, Midland.
** Bio-Rad Laboratories, Richmond.
*** LKB-Produkter AB, Stockholm.
§ C. Desaga GmbH, Heidelberg.

Trennung von Basen und Nucleosiden mit Ammoniumformiatpuffern (pH 2–4) und 0.35 M NaCl

Im ersten Diagramm sind die Ergebnisse der Trennungen in Abhängigkeit vom pH-Wert zusammengestellt. Das Retentionsvolumen von Adenin wurde = 1 gesetzt und alle anderen Retentionswerte darauf bezogen. Adenin ist sicher keine ideale Bezugssubstanz, da es zwischen pH 3 und 4 nur teilweise ionisiert vorliegt und dadurch selbst ein pH-abhängiges Retentionsvolumen aufweist; z.B. wird Adenin bei pH 4 nach etwa 300 ml und bei pH 2 nach 600 ml eluiert. Eine ideale Bezugssubstanz dürfte nicht durch pH-Veränderungen beeinflusst werden.

Die grössten Unterschiede der Retentionswerte liegen zwischen den pH-Werten 3 und 4. Hier lassen sich aus dem Diagramm die günstigsten Puffer für die Trennung verschiedener Kombinationen von Verbindungen herausfinden (Fig. 1 und 3). So kann zwischen den pH-Werten 3.5 und 3.7 die grösste Anzahl von Verbindungen getrennt werden. Aber auch in diesem Bereich fallen die Retentionswerte verschiedener Substanzen zusammen, z.B. Guanin und Cytidin, sowie 7-Methyl-adenin und 1-Methyl-adenin. Das erste Paar lässt sich bei einem pH-Wert von 3.0 trennen und das letztere bei 4.0. Im unteren pH-Bereich sind die Aminogruppen vieler Verbindungen mit Ausnahme des Guanosins vollständig ionisiert. Da keine Ladungsänderung eintreten kann, verlaufen die Retentionskurven fast parallel. Die Retentionswerte für Guanin, Adenosin, Cytosin, 5-Methyl-cytosin, 7-Methyl-adenin und 1-Methyl-adenin liegen eng beieinander. Wenn für die Trennung der Basen und Nucleoside lediglich die Ladung verantwortlich wäre, müssten auch Cytidin, Adenin, 6-Methyl-adenin und 6-Dimethyl-adenin praktisch die gleichen Retentionswerte (pH 2) besitzen wie die vorher genannten Verbindungen.

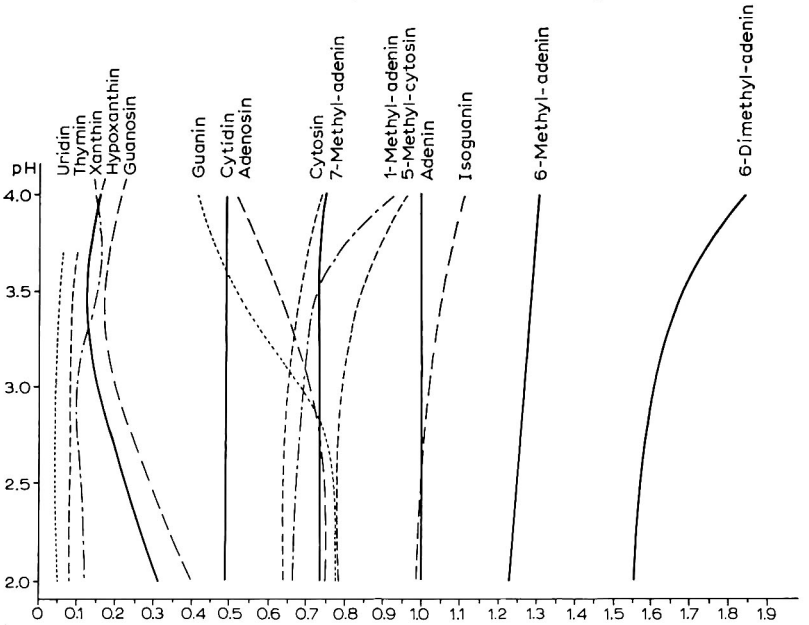

Fig. 1. Relative Retentionswerte verschiedener Basen und Nucleoside in Abhängigkeit vom pH-Wert. Das Retentionsvolumen von Adenin wurde als Bezugssubstanz = 1 gesetzt. (Dowex 50W X4, 200–400 mesh, Na^+, Säulenlänge 20 cm, Durchmesser 1 cm, Ammoniumformiatpuffer mit 0.35 *M* NaCl.)

Aus dem Diagramm (Fig. 1) mit den relativen Retentionswerten allein lassen sich die Trennmöglichkeiten nicht ablesen. Die einzelnen Verbindungen haben ein bestimmtes Elutionsvolumen, das vom Retentionswert abhängt. Alle Substanzen, die früh eluiert werden, besitzen ein kleines Elutionsvolumen von 15–30 ml, Verbindungen, die nahe beim Adenin eluiert werden eines von 90–180 ml und die letzten Substanzen eines von 200–300 ml. Deshalb wurden im zweiten Diagramm die einzelnen Elutionsvolumina bei verschiedenen pH-Werten aufgezeichnet, wie sie sich auf einer 50 cm langen Säule ergeben (Fig. 2). Die Elutionsvolumina entsprechen auch den von kürzeren Säulen. Es wurde gewöhnliches Harz, Dowex 50W X4, 200–400 mesh verwendet. Bei Benutzung von sphärischen Harzen mit ziemlich einheitlicher Partikelgrösse, lassen sich die Trennergebnisse wesentlich verbessern. Es kam uns aber darauf an mit der üblichen und gebräuchlichen Ausrüstung die bestmöglichen Ergebnisse zu erzielen, um in Routinebetrieb gute, reproduzierbare Ergebnisse zu erhalten. Höher vernetzte Harze bedingen erheblich grössere Elutionsvolumina und verschlechtern deshalb die Trennleistung.

Die Trennungen wurden alle bei Raumtemperatur (21–23°) ausgeführt. Chromatographiert man bei 37.5°, verändern sich viele Retentionswerte (Tabelle I), denn

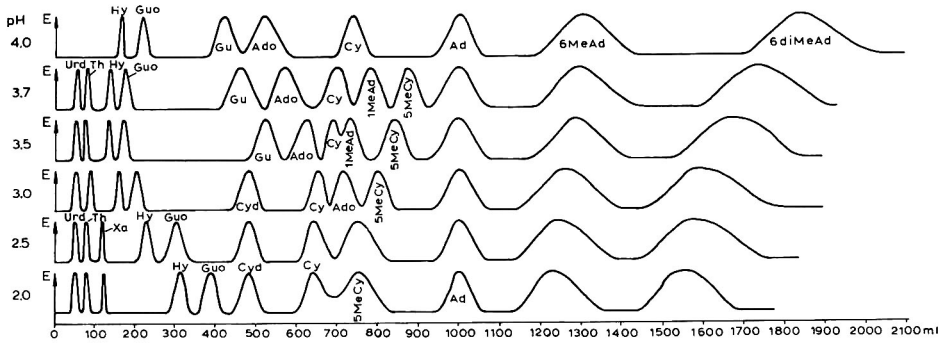

Fig. 2. Elutionsvolumen verschiedener Verbindungen im pH-Bereich 2–4. Die Trennungen wurden auf einer 50 cm langen Säule ausgeführt. Auch mit kurzen Säulen findet man die gleichen Elutionsvolumina. (Dowex 50W X4, Na^+, 200–400 mesh, Ammoniumformiatpuffer mit 0.35 *M* NaCl.)

TABELLE I

DIE RELATIVEN RETENTIONSWERTE BEZOGEN AUF ADENIN BEI VERSCHIEDENEN TEMPERATUREN (pH 3.5)

Substanz	*21–23°*	*37.5°*	*Differenz in %*
Hypoxanthin	0.12	0.18	+50
Guanosin	0.17	0.20	+18
Cytidin	0.48	0.49	+ 2
Guanin	0.52	0.49	− 6
Adenosin	0.64	0.56	−12
Cytosin	0.69	0.77	+11
1-Methyl-adenin	0.72	0.83	+15
5-Methyl-cytosin	0.82	0.93	+13
Adenin	1.00	1.00	± 0
6-Methyl-adenin	1.29	1.35	+ 5
6-Dimethyl-adenin	1.68	1.71	+ 1.5

höhere Temperaturen begünstigen die Desorption. So erhöht sich der Retentionswert des Cytosins von 0.69 auf 0.77 und der des Methylcytosins von 0.82 auf 0.93 (Tabelle I). Aber auch einige Purine werden später eluiert, so dass man keine durchgehende Regel aufstellen kann. Gleichzeitig werden die einzelnen Banden schärfer, das heisst, sie besitzen ein kleineres Elutionsvolumen, und damit verbessert sich die Trennleistung.

Hypoxanthin und Guanin werden bei tiefen pH-Werten deutlich später eluiert als bei hohen pH-Werten. Man kann diesen Effekt für eine Abtrennung von Hypoxanthin und Guanosin ausnutzen. Im nächsten Abschnitt werden jedoch für diese Verbindungen bessere Trennmöglichkeiten beschrieben.

Trennung von Basen und Nucleosiden mit Natriumformiatpuffer (pH 2–3) und 0.05 M Na^+

Substanzen mit dem Elutionswert von 0.3 lassen sich zwar als Reinsubstanzen gut trennen, jedoch nicht aus biologischem Material. Hier kommen in den ersten 100 ml zahlreiche organische Substanzen mit U.V.-Absorption oder nach Aufgabe von sauer hydrolysierten Extrakten Bräunungsprodukte, die eine quantitative Bestimmung zu Beginn der Elution unmöglich machen. Wir haben deshalb Harnsäure, Inosin, Hypoxanthin und Guanosin mit Natriumformiatpuffer (0.05 *M* Na^+) eluiert. Mit diesem Elutionsmittel kommen die Substanzen wesentlich später. Hypoxanthin und Guanosin werden bei pH 2.5 und 2.0 (Fig. 3) schon so spät eluiert, dass sich die chromatographische Trennung unnötig verzögert. Man kann natürlich auch beide Elutionsmittel kombinieren, und nach der Trennung von Inosin, Hypoxanthin und Guanosin mit der Salzkonzentration von 0.35 Mol und dem entsprechenden pH-Wert die übrigen Substanzen eluieren.

Harnsäure oder Xanthin und Inosin als Reinsubstanzen lassen sich zwar abtrennen, aber in biologischem Material oft nicht quantitativ messen. Wir haben deshalb ein Verfahren ausgearbeitet, das einen grossen Teil der Abbauprodukte des Purinstoffwechsels auf einer Säule trennt.

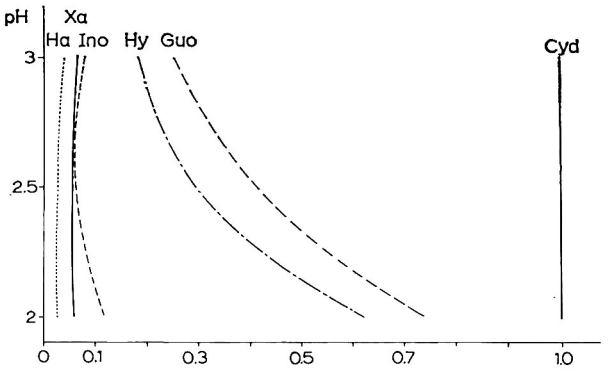

Fig. 3. Relative Retentionswerte einiger Basen und Nucleoside im pH-Bereich von 2–3. Cytidin diente als Bezugssubstanz. Der Natriumformiatpuffer besitzt eine Ionenkonzentration von 0.05 *M* Na^+. (Dowex 50W X4, Na^+, 200–400 mesh, Säulenlänge 20 cm, Durchmesser 1 cm.)

Trennung mit H_2O und HCl als Elutionsmittel

Auf Kationenaustauschern (Dowex 50W X4, 200–400 mesh) in der H^+-Form eluierten wir NAD^+ und 5'-AMP mit H_2O, Xanthin und Inosin mit 0.005 *N* HCl und

anschliessend mit 0.5 *N* HCl Hypoxanthin, Nicotinsäureamid und Adenosin. Danach kann man mit 2 *N* HCl[5] auch Adenin und Guanin trennen. Die Fig. 4 zeigt die Trennung eines Gewebsextraktes. Für die Bestimmung der Abbauprodukte von Purin nucleotiden in Herzgewebe hat sich die Methode bewährt, jedoch ist das mehrmalige Wechseln des Elutionsmittels und auch die lange Elutionsdauer umständlich.

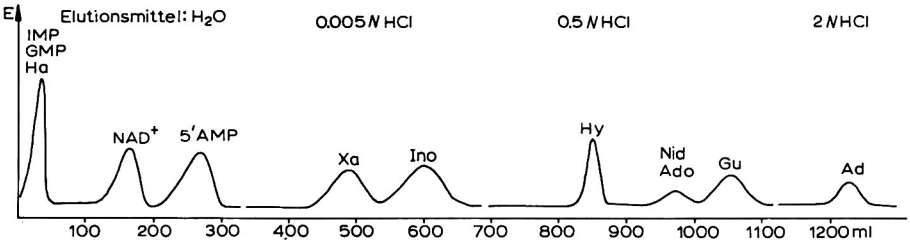

Fig. 4. Trennung von Nucleosiden, Nucleotiden und Basen auf Kationenaustauschern. Elution mit Wasser und steigenden Konzentrationen von HCl-Lösungen. (Dowex 50W X4, H^+, 200–400 mesh, Säulenlänge 25 cm, Durchmesser 1 cm.)

Trennung von Nucleotiden auf Kationenaustauschern

Auf Dowex 50W X4, in der H^+-Form lassen sich Mononucleotide von den entsprechenden Deoxynucleotiden trennen (Fig. 5), mit Ausnahme von Thymidinmonophosphat und Uridylsäure. Als Elutionsmittel dient 0.001 *N* HCl. Die Nucleotide besitzen in diesem Bereich als Zwitterionen, abgesehen von ThMP und UMP, verschieden stark ionisierte Aminogruppen und unterscheiden sich daneben in ihrer Adsorption an die Matrix des Austauschers[5]. Beide Effekte machen die Trennung aus. Etwa 10–20 % der Purindeoxynucleotide werden auf der Säule N-glykosidisch gespalten[4,17]. Die 2',3'-Isomere konnten mit der gleichen Anordnung nur teilweise getrennt werden, da 2',3'-CMP mit AMP zusammenfiel. Es liess sich lediglich UMP, 2',3'-GMP und 2',3'-AMP gut trennen (Fig. 6). KATZ UND COMB[18], sowie BLATTNER UND ERIKSON[19], die eine ähnliche Trennung für 2',3'-Ribonucleotide beschrieben, konnten AMP und CMP auf Säulen mit normaler Länge auch nicht separieren.

Erfolgreicher waren die Versuche, die verschiedenen Adeninnucleotide zu

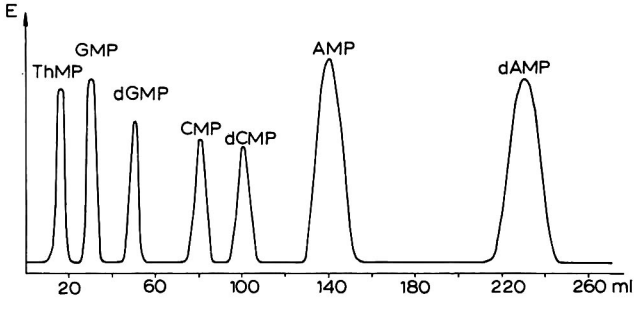

Fig. 5. Trennung von Nucleotiden und Deoxynucleotiden auf Kationenaustauschern. (Dowex 50W X4, Na^+, −400 mesh, Säulenlänge 55 cm, Durchmesser 1 cm, Elutionsmittel 0.001 *N* HCl + 0.01 *M* NaCl.)

trennen. Zu diesem Zweck benutzten wir wiederum Kationenaustauscher in der H^+-Form. Auf einer 20 cm langen Säule lassen sich $NADP^+$, zyklisches AMP, 2',3'- und 5'-AMP trennen (Fig. 7). Als Elutionsmittel diente 0.1 *N* HCl. NAD^+ fällt jedoch mit dem 3'-AMP zusammen (Fig. 7). Die Nucleotide wurden fast alle als Reinsubstanzen getrennt, grössere Erfahrungen mit Bestimmungen aus biologischem Material liegen nicht vor im Gegensatz zu allen anderen beschriebenen Verfahren.

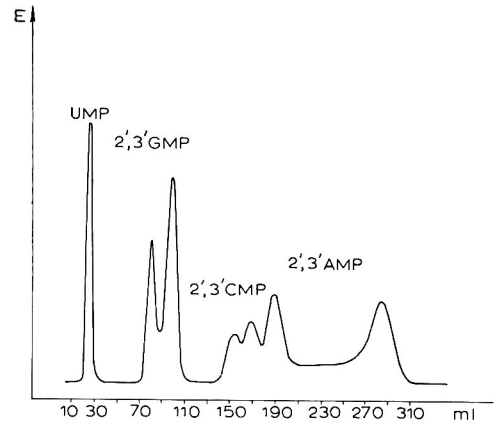

Fig. 6. Trennung von 2',3'-Nucleotiden auf einem Kationenaustauscher. (Dowex 50W X4, H^+, −400 mesh, Säulenlänge 15 cm, Durchmesser 1 cm, Elutionsmittel 0.01 *N* HCl.)

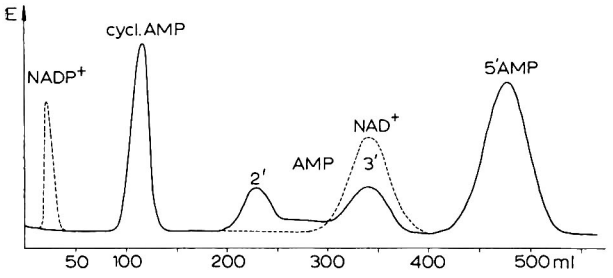

Fig. 7. Trennung von Adenosinnucleotiden auf Kationenaustauschern. (Dowex 50W X4, H^+, 200–400 mesh, Säulenlänge 20 cm, Durchmesser 1 cm, Elutionsmittel 0.01 *N* HCl.) Die Retentionsvolumina der Substanzen mit den punktierten Linien wurden in separaten Versuchen ermittelt.

Anwendung

Diese Methoden wurden für Arbeiten mit biologischem Material entwickelt und alle mit Ausnahme der Trennung der Nucleotide im Routinebetrieb eingesetzt. Wir möchten daher einige von uns routinemässig angewandte Trennungen beschreiben, z.B. die Bestimmung der vier DNS-Basen aus Nieren- und Hodengewebe. Der Rückstand nach alkalischer Hydrolyse wird 1 Std. mit 10 *N* $HClO_4$ hydrolysiert. Das stark schwarz gefärbte Hydrolysat kommt nach Abstumpfung und Verdünnung direkt auf die Säule. Die einzelnen Komponenten liessen sich ohne Schwierigkeiten trennen und

quantitativ bestimmen, wie aus der Fig. 8 deutlich wird. Auch die Purinbasen und Pyrimidindeoxynucleoside nach enzymatischer DNS-Spaltung lassen sich gut trennen[9].

Wesentlich schwieriger war die Aufgabe, den Abbau der Purinnucleotide im Gewebe zu verfolgen. Die Konzentrationen der gesuchten Substanzen sind sehr klein und mit zahlreichen U.V.-absorbierenden Substanzen vermischt[11].

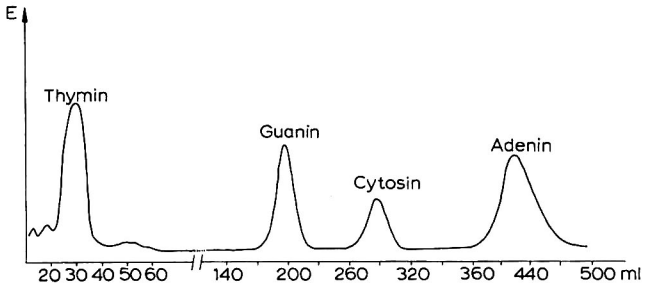

Fig. 8. Säurehydrolysat des Rückstandes von Nierengewebe nach einer Aufarbeitung von Schmidt-Thannhauser (Bestimmung der Basen aus DNS). (Dowex 50W X4, Na^+, −400 mesh, Säulenlänge 20 cm, Durchmesser 1 cm, Ammoniumformiatpuffer pH 3.5 + 0.35 *M* NaCl.)

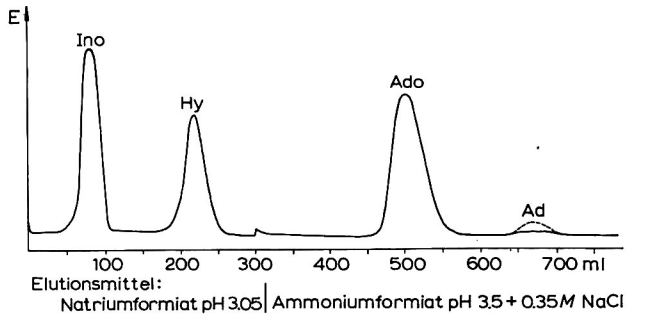

Fig. 9. Inkubation von gewaschenen Erythrocyten mit Adenosin. Inosin und Hypoxanthin wurden mit einem Natriumformiatpuffer pH 3.0 (0.05 *M* Na^+), Adenosin und Adenin mit einem Ammoniumformiatpuffer von pH 3.5 + 0.35 *M* NaCl getrennt. (Dowex 50 W X4, Na^+, −400 mesh, Säulenlänge 20 cm, Durchmesser 1 cm.)

Die Fig. 9 zeigt die Trennung eines Extraktes aus Inkubationsversuchen mit Erythrocyten und Adenosin. Zuerst werden Inosin und Hypoxanthin und nach Pufferwechsel Adenosin und Adenin getrennt. Die verschiedenen beschriebenen Möglichkeiten sind weitgehend aus dem Zwang entstanden, für verschiedene Gewebe gute und quantitativ auswertbare Trennungen zu bekommen. Es können grosse Extraktmengen aufgetragen werden, bis zu 30 ml, entsprechend 3–5 g Gewebe. Geringe Konzentrationen kann man daher genau und zuverlässig bestimmen ohne die Extrakte vorher einzuengen.

In den oben erwähnten Inkubationsversuchen mit Erythrocyten und Adenosin fanden wir neben den erwarteten Spaltprodukten Inosin und Hypoxanthin auch

geringe Mengen an Adenin. Die Frage, ob dieses Adenin nicht als Beimengung im Adenosin bereits vorhanden war, veranlasste uns, verschiedene käufliche Basen und Nucleoside auf ihre Reinheit zu untersuchen. Wir konnten auf die Säule bis zu 30 mg auftragen und dadurch noch Beimengungen von 0.1 % gut bestimmen. Die Ergebnisse sind in der Tabelle II zusammengestellt. Die Präparate haben nur geringe Beimengungen von 0.2–0.9 %, mit Ausnahme einer Inosinprobe (Tabelle II).

TABELLE II

Präparat	*Chromatographisch bestimmte Basen (Angaben in Mol-%)*
Inosin[a]	2.3% Hypoxanthin
Inosin[b]	0.2% Hypoxanthin
Guanosin[b]	0.9% Guanin
Adenosin[a]	0.25% Adenin

[a] C. F. Boehringer und Soehne GmbH, Mannheim.
[b] Sigma Chemical Company, St. Louis.

ZUSAMMENFASSUNG

Es wurden die Trennungen und Bestimmungen von Basen und Nucleosiden auf Kationenaustauschern mit Ammoniumformiatpuffern systematisch untersucht und die Ergebnisse in Diagrammen zusammengefasst. Weitere Trennmöglichkeiten mit H_2O und verdünnter HCl wurden beschrieben. Alle Verfahren benutzten wir im Routinebetrieb. Über Trennungen von Nucleotiden (nur Reinsubstanzen) auf Kationenaustauschern wird ebenfalls berichtet.

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CHROM. 3697

SÉPARATION PAR ÉLECTROPHORÈSE SUR PAPIER DE Ge^{IV}, As^{III}, As^{V} ET DE Ge^{IV}, $^{77}As^{III}$, $^{77}As^{V}$

ÉTUDE CINÉTIQUE DE L'OXYDATION DU RADIOARSENIC PAR LE PEROXYDE D'HYDROGÈNE

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(Reçu le 17 juillet 1968)

SUMMARY

Separation of Ge^{IV}, As^{III}, As^{V} and Ge^{IV}, As^{III}, As^{V} by paper electrophoresis. Kinetic study of the oxidation of radioactive arsenic by hydrogen peroxide

A method has been developed for the separation of macro amounts of As^{0}, Ge^{IV}, As^{III} and As^{V} by paper electrophoresis. When using tracer amounts of radioactive products, the solution to be analysed should contain macro quantities of arsenite, otherwise the trace of trivalent radioarsenic will be oxidized during the separation.

The method has been applied to the study of the reaction:

$$^{77}As^{III} + H_2O_2 \xrightarrow{k_1} {}^{77}As^{V} + 2\ OH^-$$

and has enabled us to determine its rate constant at pH 14, which was found to be

$$k_1 = 2.3 \cdot 10^2\ M^{-1} \cdot sec^{-1}$$

Au cours d'une étude sur les effets physico-chimiques associés aux processus nucléaires[1]:

$$^{76}Ge(n,\gamma)^{77}Ge \xrightarrow{\beta^-} {}^{77}As \xrightarrow{\beta^-}$$

nous avons été amenés à séparer une quantité pondérable d'ions germanates marqués, d'une quantité impondérable de radioarsenic susceptible de se trouver sous forme neutre ou sous forme d'ions trivalents ou pentavalents. On s'est, de plus, proposé de séparer et doser ces différents états de valence du radioarsenic.

SÉPARATION DE Ge^{IV}, As^{III}, As^{V}

Tout d'abord, nous avons mis au point une méthode d'analyse par électrophorèse sur papier, qui nous permet de séparer en quantités pondérables les différentes espèces qui nous intéressent.

Les solutions à analyser sont Ge^{IV} 0.2 M, As^{III} 10^{-2} M et As^{V} 10^{-2} M, en milieu KOH 1.4 N. La préparation de solutions relativement concentrées d'ions germanates à partir de GeO_2 nécessite que le milieu de dissolution soit basique. Il faut noter que la difficulté majeure consiste à séparer Ge^{IV} et As^{III} qui, dans nos conditions de travail, ont un comportement électrophorétique voisin.

La séparation se fait, sur 20 mm^3 de solution, par électrophorèse sur papier dans des cuves Jouan légèrement modifiées (Fig. 1). On utilise des bandes de papier Whatman 3 MM (39 × 3 cm) imprégnées d'une solution de potasse 0.05 N qui constitue l'électrolyte.

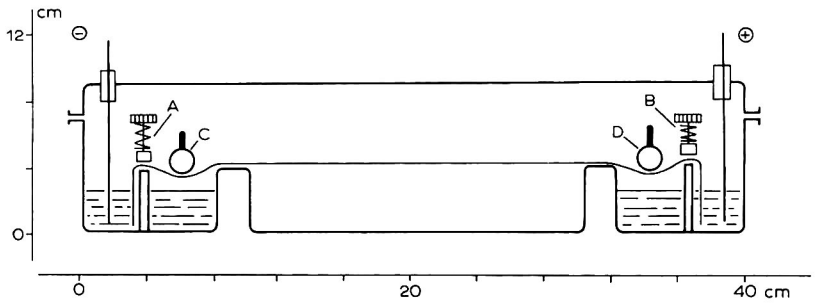

Fig. 1. Cuve employée pour l'électrophorèse sur papier. A et B = ressorts. C et D = contrepoids.

Précisons que pour éviter la carbonatation rapide de cet électrolyte, les solutions sont préparées immédiatement avant chaque séparation. Nous avons dû prendre ces précautions, car la présence de traces de carbonate perturbe fortement la séparation entre les ions germanates et arsénites. On applique pendant une heure une tension continue de 600 V; dans ces conditions, les vitesses de déplacement des différents ions sont:

Germanate: 11.5 $cm \cdot h^{-1}$
Arsénite: 15 $cm \cdot h^{-1}$
Arséniate: 20 $cm \cdot h^{-1}$

Les positions des ions inactifs sont localisées par des réactifs colorés, la quercétine dans l'alcool pour les germanates (couleur orange) et le nitrate d'argent en solution aqueuse pour les arsénites (couleur jaune) et les arséniates (teinte ocre).

Cette méthode analytique nous permet d'obtenir la séparation de Ge^{IV}, As^{III}, As^{V}. Ces trois formes ioniques sont chargées négativement en milieu basique et elles se trouvent séparées suivant l'ordre indiqué.

ÉTUDE DES ÉTATS DE VALENCE DE L'ARSENIC-77

Cette séparation a été utilisée pour résoudre un problème radiochimique précis. En effet, après irradiation au réacteur nucléaire, GeO_2 referme dans son réseau du radioarsenic provenant de la désintégration β^- à la fois de ^{77m}Ge et de ^{77}Ge. Il s'agit de déterminer dans quels états de valence se trouve l'arsenic-77. Ceci nous amène, tout d'abord, à préciser les conditions d'obtention et de mesure des différents radio-éléments mis en jeu.

Activation au réacteur nucléaire

Par irradiation de GeO_2 au réacteur nucléaire pendant 40 h à un flux de $2.2 \cdot 10^{12}$ n·cm^{-2}·sec^{-1}, trois des cinq isotopes stables du germanium s'activent (Tableau I).

Dix heures après l'irradiation les seules activités β^- mesurables sont celles de ^{77}Ge et ^{77}As.

TABLEAU I

Isotope	*Pourcentage isotopique*	*Section efficace isotopique*	*Processus nucléaires*
^{70}Ge	20.55	3.9 b	$^{70}Ge(n,\gamma)^{71}Ge \xrightarrow[11\ j]{C.E.} {}^{71}Ga$ stable
^{74}Ge	36.74	0.25 b	$^{74}Ge(n,\gamma)^{75m}Ge \xrightarrow[48\ sec]{T.I.} {}^{75}Ge \xrightarrow[82\ min]{\beta^-} {}^{75}As$ stable
			$^{74}Ge(n,\gamma)^{75}Ge \xrightarrow[82\ min]{\beta^-} {}^{75}As$ stable
^{76}Ge	7.67	0.14 b	$^{76}Ge(n,\gamma)^{77m}Ge(53\ sec) \xrightarrow[\sim 75\%]{\beta^-} {}^{77}As \xrightarrow[38.8\ h]{\beta^-} {}^{77}Se$ stable
			T.I. $\downarrow \sim 25\%$
			$^{76}Ge(n,\gamma)^{77}Ge \xrightarrow[11.4\ h]{\beta^-} {}^{77}As \xrightarrow[38.8\ h]{\beta^-} {}^{77}Se$ stable

Détection et mesure des rayonnements

Afin de connaître avec précision la répartition de l'activité du radioarsenic entre les différentes formes $^{77}As^0$, $^{77}As^{III}$ et $^{77}As^V$, nous avons spécialement adapté un ensemble de détection et de comptage. Nous disposons d'un dérouleur de chromatogramme associé à un détecteur, qui peut être, soit un tube de Geiger-Müller à fenêtre mince (1.8 mg/cm^2) soit un cristal d'anthracène monté sur un tube photomultiplicateur. Dans le second cas la chaîne de comptage fonctionne en sélecteur d'amplitude, la position discriminateur est réglée avec un seuil tel qu'on élimine le comptage des rayons X mous dus à la capture électronique de ^{71}Ge. La nature du détecteur est choisie en fonction du problème que pose le comptage du chromatogramme. Aussi bien en Geiger-Müller qu'en compteur à scintillations, le signal issu du détecteur attaque simultanément deux intégrateurs; l'un commande l'enregistreur, l'autre l'échelle de comptage par l'intermédiaire d'un relais, qui déclenche l'arrêt-marche de cette échelle lorsque l'activité instantanée est supérieure à une fois et demie le mouvement propre.

Le dépouillement de nos résultats se réalise de la façon suivante: le pourcentage des différentes activités est calculé en faisant le rapport du taux de comptage de chaque pic (proportionnel à la surface du pic), à l'activité totale du radioarsenic contenu sur la bande de papier. Le mouvement propre intégré en coups par centimètre est calculé et soustrait d'après le temps de passage du pic.

Dans la mesure du possible, on s'arrange toujours pour que les activités instan-

tanées soient mesurables sur la gamme de sensibilité: 150 impulsions par seconde des intégrateurs, d'une part pour avoir une bonne précision sur le taux de comptage, d'autre part pour que le tube de Geiger-Müller ne décompte pas. Nous avons vérifié que les pourcentages déterminés par les mesures de surfaces de nos pics, au planimètre, et ceux obtenus par les taux de comptage se recoupent à $\pm$ 1 % près. D'une manipulation à l'autre, les résultats sont reproductibles à $\pm$ 2 %.

Séparation des espèces actives

Les solutions à analyser sont préparées par dissolution de 20 mg de GeO_2 irradié au réacteur nucléaire dans 1 cm³ de solution KOH 1.4 *N*, 10^{-2} *M* en As^{III} et 10^{-2} *M* en As^{V}. La séparation par électrophorèse sur papier est réalisée dans les mêmes conditions d'analyse que celles définies pour les espèces inactives, mais cette fois la position des ions est repérée par leur radioactivité (Fig. 2) à l'aide de l'appareillage décrit précédemment.

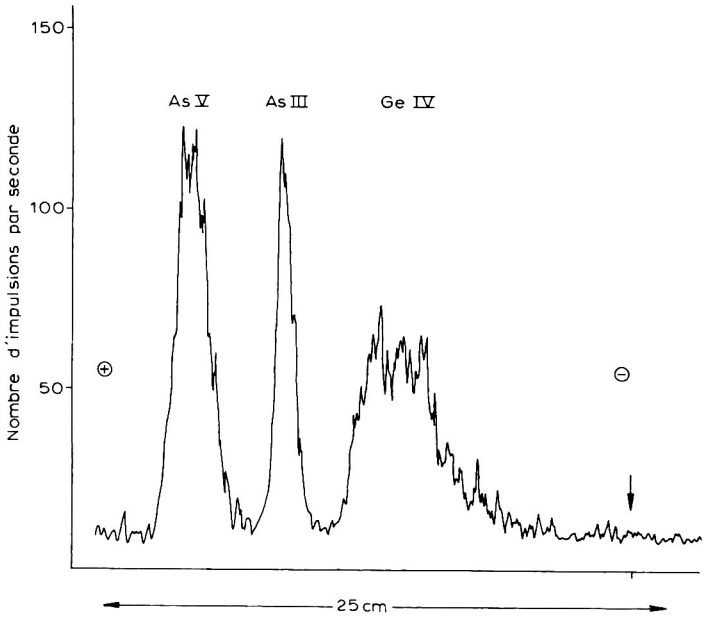

Fig. 2. Enregistrement d'un radiochromatogramme. La flèche indique l'endroit du dépôt des 20 mm³ de solution à analyser.

Ainsi, nous déterminons simultanément le pourcentage relatif de $^{77}As^{III}$ et $^{77}As^{V}$. Et par exemple, dans les conditions où nous nous sommes placés, c'est-à-dire mise en solution de GeO_2 irradié au réacteur en présence d'entraîneurs de l'arsenic, nous sommes conduits à 85 % $^{77}As^{III}$ et 15 % $^{77}As^{V}$.

En absence d'entraîneurs (As^{III}) les ions arsénites radioactifs ($^{77}As^{III}$) sont entièrement oxydés pendant la séparation. Pour protéger le radioarsenic trivalent contre cette oxydation pendant l'électrophorèse, il faut que la solution à analyser soit au moins 10^{-3} *M* en arsénite (nous avons vérifié qu'une concentration de

10^{-4} M en arsénite est insuffisante pour empêcher l'oxydation spontanée de $^{77}As^{III}$). Toutes nos séparations sont donc effectuées en amenant juste avant l'électrophorèse les solutions à une concentration d'environ $3 \cdot 10^{-2}$ M en As^{III}. Pour le radioarsenic pentavalent, la présence ou l'absence d'entraîneurs dans la solution à analyser ne modifie pas de façon importante sa position sur le chromatogramme. De plus, nous avons vérifié, avec du radioarsenic élémentaire obtenu par réduction cathodique en milieu acide des ions arsénieux radioactifs, que l'espèce As^0 ne migre pas ou migre peu.

APPLICATION À L'ÉTUDE DE LA RÉACTION $^{77}As^{III} + H_2O_2$

Les limites de validité de la méthode analytique étant précisées à l'échelle des indicateurs, nous avons entrepris de déterminer la valeur de la constante de vitesse de la réaction:

$$^{77}As^{III} + H_2O_2 \longrightarrow {}^{77}As^{V} + 2\ OH^-, \qquad (1)$$

en milieu alcalin.

L'oxyde de germanium utilisé pour préparer les solutions de radioarsenic trivalent sans entraîneurs est recuit, après son irradiation au réacteur nucléaire, pendant 1 h à 750°, avant d'être dissous. Ces précautions sont prises de façon à éliminer le rôle joué par les défauts au moment de la mise en solution de l'oxyde irradié[2].

Le peroxyde d'hydrogène est préparé synthétiquement par irradiation aux rayons γ du radiocobalt, de solution aérée d'eau tridistillée pendant 20 ou 40 h à un débit de dose de 10^{19} $eV \cdot cm^{-3} \cdot h^{-1}$.

Nous avons ainsi suivi l'oxydation d'ions radioarsenic trivalents en solution alcaline KOH 1.4 N, à 20°, par du peroxyde d'hydrogène $1.4 \cdot 10^{-5}$ M. La réaction est bloquée, à intervalles de temps réguliers, sur chaque partie aliquote, par addition d'une quantité pondérable d'arsénite de façon à obtenir pour l'analyse une solution $3 \cdot 10^{-2}$ M en As^{III}. Nous sommes alors conduits à une courbe cinétique classique (Fig. 3).

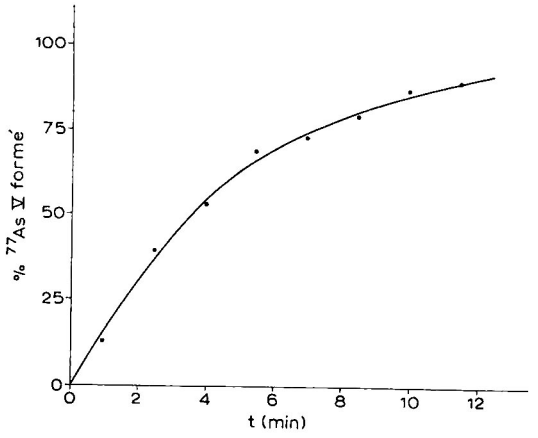

Fig. 3. Courbe cinétique de l'oxydation de $^{77}As^{III}$ "sans entraîneur" en solution KOH 1.4 N par du peroxyde d'hydrogène $1.4 \cdot 10^{-5}$ M à 20°.

En portant le logarithme de la quantité de $^{77}As^{III}$ restant à oxyder en fonction du temps, nous obtenons une droite. On est donc bien ramené à une cinétique du premier ordre comme on pouvait s'y attendre d'après le rapport des concentrations des espèces réagissantes. La constante de vitesse de la réaction (1) calculée d'après la courbe cinétique est de:

$$k_1 = 2.3 \cdot 10^2 \, M^{-1} \cdot \sec^{-1}.$$

Cette réaction est donc plus rapide en milieu basique qu'en milieu acide puisque WOODS, KOLTHOFF ET MEEHAN[3] ont déterminé qu'en milieu $HClO_4$ 0.1 N:

$$k'_1 = 1.01 \cdot 10^{-2} \, M^{-1} \cdot \sec^{-1}.$$

RÉSUMÉ ET CONCLUSION

Une méthode d'électrophorèse sur papier a été mise au point; elle permet de séparer en quantité pondérable As^0, Ge^{IV}, As^{III}, As^V. Lorsque l'on se place à l'échelle des indicateurs en utilisant des produits radioactifs, il est indispensable que la solution à analyser contienne une quantité pondérable d'arsénite, sinon le radioarsenic trivalent impondérable se trouve oxydé pendant la séparation.

L'application de cette méthode analytique à l'étude de la réaction:

$$^{77}As^{III} + H_2O_2 \xrightarrow{k_1} {}^{77}As^V + 2\ OH^-$$

nous a permis de déterminer, à pH 14, la valeur de la constante de vitesse de cette réaction:

$$k_1 = 2.3 \cdot 10^2 \, M^{-1} \cdot \sec^{-1}.$$

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Notes

CHROM. 3731

Über einen neuen kontrastfähigen Fluorescenzindikator für die Dünnschichtchromatographie der Lipide

Zur Sichtbarmachung der Flecken von Lipidgemischen sind bei der Dünnschichtchromatographie zahlreiche Fluorescenzindikatoren in Gebrauch: Rhodamin B, Fluorescein, Dichlorfluorescein u.a. Ihnen haftet der gemeinsame Mangel an, dass sie die Flecken nicht scharf genug hervorstechen lassen und infolgedessen keine brauchbaren, d.h. scharf umrissenen, schwarz-weissen oder gar farbigen Bilder liefern können. Wir prüften unterschiedliche im U.V.-Licht fluorescierende chemische Verbindungen, die bislang als Indikatoren in der Dünnschichtchromatographie nicht eingesetzt wurden, und stellten hierbei fest, dass einige Diaminostilbentriazin- und Diphenylpyrazolin-Derivate, die in der Textilindustrie als optische Aufheller Verwendung finden, sich für unseren Zweck am besten eignen.

Experimenteller Teil

Zur Untersuchung gelangten nachstehende optische Aufheller der Firma Geigy (Basel) unter der Handelsbezeichnung Tinopal: RBS, GS, CWS, TAS, LCS, 4BMS und CH 3566.

Die Voruntersuchungen zur Feststellung des nachweisbaren Minimums an Lipiden ergaben, dass bei den Tinopalen RBS, GS, CWS und TAS die Flecken nicht scharf genug zum Fond kontrastieren oder dass ihre Empfindlichkeit über 10 γ Substanz liegt. Aus diesem Grunde mussten sie aus den weiteren Untersuchungen ausscheiden, die wir dann mit LCS und 4BMS (Diaminostilbentriazin-Derivate) sowie mit CH 3566 (Diphenylpyrazolin-Derivat) durchführen. Wir benützten die Tinopalen als 0.5%-ige alkoholische Lösungen.

Das Chromatogramm eines Lipidgemisches mit der Zusammensetzung 4 γ *n*-Oktadekan, 6 γ Tristearat, 3 γ Stearinsäure, 2 γ Palmitinalkohol und 2 γ Phytosterole in jedem Auftragspunkt ist in Fig. 1 dargestellt. Die Flecke machten wir im U.V.-Licht mit folgenden Indikatoren sichtbar: (1) Tinopal LCS, (2) Tinopal CH 3566, (3) Dichlorfluorescein, (4) Fluorescein und (5) Rhodamin B.

Offensichtlich ist die Empfindlichkeit des Fluoresceins als Indikator für Lipide sehr geringfügig, während die des Rhodamins B über 3 γ Substanz liegt. Analoge Versuche, doch mit kleineren Mengen des Lipidgemisches, ergaben, dass die gerade noch nachweisbare Menge an Lipiden für das Dichlorfluorescein, für CH 3566 und LCS bei 1.5 bis 3.0 γ liegt.

In Anlehnung an die Versuche von JONES und Mitarb.[1], nach denen der Zusatz von Rhodamin B zum Dichlorfluorescein dessen Empfindlichkeit weitgehend steigert, stellten wir eine Reihe von Versuchen mit Zusatz von Rhodamin B zu Tinopalen an. In Fig. 2 ist das gleiche Lipidgemisch wie in Fig. 1, doch in einer um die Hälfte ver-

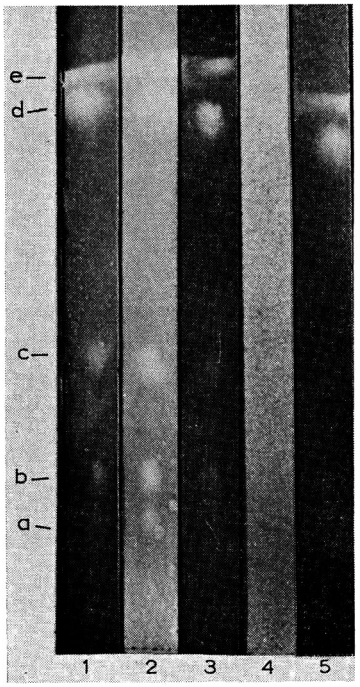

Fig. 1. Chromatogramm eines Lipidgemisches. Adsorbent: Silicagel + 16% Gips. Laufmittel: Hexan–Aceton (9:1). a = Phytosterole; b = Palmitinalkohol; c = Stearinsäure; d = Tristearat; e = Oktadekan. 1–5, Sichtbarmachung, s. Tekst.

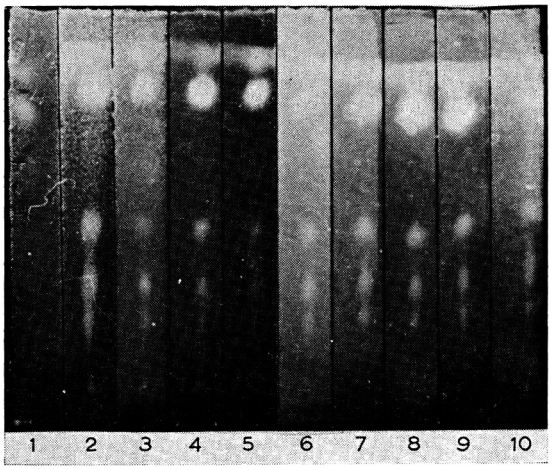

Fig. 2. Chromatogramm des gleichen Lipidgemisches unter denselben Voraussetzungen wie in Fig. 1, doch sind die Flecken gefärbt mit: Dichlorfluorescein (1); 0.5%iger Lösung von CH 3566 mit Zusatz von Rhodamin B, 20 mg/g (2), 10 mg/g (3), 30 mg/g (4); 0.5%-iger Lösung von 4BMS mit Rhodamin B-Zusatz, 30 mg/g (5), 10 mg/g (6), 20 mg/g (7); 5%iger Lösung von LCS mit Rhodamin B-Zusatz, 10 mg/g (8), 30 mg/g (9) und 20 mg/g (10).

ringerten Menge, chromatographiert. Das Fliessmittel ist in beiden Fällen dasselbe.

Der Zusatz von Rhodamin B zum jeweiligen Fluorescenzindikator lässt, wie ersichtlich, die Flecken schärfer vom Fond hervorstechen. Das nachweisbare Minimum an Lipiden beträgt 0.5 bis 1.0 γ. Die Menge des zugesetzten Rhodamins B kann innerhalb der Grenzen 15 bis 40 mg je 1 g Fluorescenzindikator schwanken, ohne dass der scharfe Kontrast der Flecken zum Fond beeinträchtigt wird. Werden diese Grenzen jedoch über- bzw. unterschritten, so bewirkt das eine wesentliche Einschränkung der Indikatorempfindlichkeit.

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CHROM. 3699

Thin layer chromatographic studies on some new nitrophenothiazines

Phenothiazines have been used extensively in medicine and industry as anti-helmintics, antihistamines, dyes and antioxidants. The rapid separation and identification of nuclear substituted phenothiazines thus merits further investigation.

Although a number of references deal with the thin layer chromatographic separation of 10-substituted phenothiazines[1–8], there is no report in the literature with regard to the thin layer chromatography of nitrophenothiazines. In the present investigation we successfully applied this technique to the qualitative analysis of a number of nitrophenothiazines and nitrodiphenyl sulphides. Various solvent systems have been established that permit the detection and differentiation of these compounds.

The U.V. absorption maxima of the compounds are also reported, which may be of interest in their characterisation.

Nitrophenothiazines and diphenyl sulphides were obtained by treatment of reactive halogenonitro compounds with substituted *o*-aminothiophenols under alkaline conditions. Halogenonitrobenzenes having both positions *ortho* to the activated halogen atom substituted either by two nitro groups or by one nitro group and one halogen atom, provided the nitrophenothiazines directly. On the other hand, halogenonitrobenzenes having only one nitro group *ortho* to the activated halogen atom provided the diphenyl sulphides, which on formylation followed by SMILES rearrangement gave the respective nitrophenothiazines.

The plates (25 mm × 25 mm) were coated with Silica Gel G (10 % $CaSO_4$), and dried and activated in the usual manner.

The plate was spotted with a micropipet 0.5 cm from one end with 1–2 μl of a benzene solution containing 1–10 μg/ml test solution. The plate was then placed in a chamber saturated with the solvent, and developed by the ascending method until the solvent front had travelled 10–15 cm. The solvent was allowed to evaporate, and the colour of the spots was observed before and after exposure to iodine vapour and their locations were compared with a reference chromatogram.

It was found that silica gel washed with chloroform gave much better and reproducible R_F values than when unwashed. In a benzene–hexane system, a higher benzene content tended to increase the R_F values and a higher hexane content to decrease the R_F values. It was also observed that 3-methyl-7-nitro-, 3-chloro-7-nitro-, and 2-chloro-7-nitro-phenothiazines had lower R_F values than the 1-nitro substituted phenothiazines which exhibit a six membered chelate of high stability through strong –N–H$\cdots$O–N– bonding between the hydrogen atom of the secondary amino group and the oxygen atom of the nitro group at position 1. The presence of the hydrogen bond in 1-nitrophenothiazines was also established by I.R. spectral studies. It was noted that benzene–acetone, benzene–methanol, acetone and various systems of methanol, with or without water, did not yield good separations.

The U.V. absorption maxima of nitrophenothiazines and nitrodiphenyl sulphides are also listed in Tables I and II. These data can be profitably used for the qualitative determination of such compounds with a minimum amount of the sample,

TABLE I

R_F VALUES AND U.V. CHARACTERISTICS OF SOME NEW NITROPHENOTHIAZINES

Phenothiazine	*R_F value ($\times$ 100) for solvent**				*M.p.* °C	*Spot colour*	*Absorption max.* ($m\mu$)
	A	*B*	*C*	*D*			
1-Nitro-7-methyl-	75	67	50	40	141	violet	250, 315
1-Nitro-7-bromo-	72	64	48	35	219	violet	250, 315
1-Nitro-7-chloro-	78	68	52	42	217	light violet	250, 315
1,3-Dinitro-7-chloro-	81	72	58	45	217	red-violet	240, 300
1,3-Dinitro-8-chloro-	75	64	53	41	265	red-violet	240, 296
1,3-Dinitro-7-bromo-	70	59	50	37	210	red-violet	242, 299
1,3-Dinitro-7-methoxy-	83	73	62	53	203	violet	241, 300
1,3-Dinitro-7-methyl-	79	67	55	48	223	violet	240, 300
1-Nitro-3-chloro-7-bromo-	88	78	62	58	261	violet	251, 318
1-Nitro-3-chloro-7-methyl-	80	69	53	47	208	violet	250, 315
1-Nitro-3,7-dichloro-	70	58	42	39	203	violet	250, 315
1-Nitro-3,8-dichloro-	63	50	35	30	303	violet	255, 315
1-Nitro-3-chloro-	77	64	52	45	250	violet	250, 313
1,3-Dinitro-	69	58	43	35	148	violet	240, 295
3-Chloro-7-nitro-	47	35	25	16	268	pink	250, 285
2-Chloro-7-nitro-	44	33	22	14	244	yellow	245, 305
3-Methyl-7-nitro-	42	31	19	10	232	pink	246, 310
Phenothiazine	73	65	52	(41)	182	green**	253, 320

* Solvent systems: A = benzene; B = benzene–hexane, 70:30; C = benzene–hexane, 50:50; D = pyridine–hexane, 10:90.

** Turns green after exposure to iodine vapours.

TABLE II

R_F VALUES AND U.V. CHARACTERISTICS OF SOME NEW NITRODIPHENYL SULPHIDES

Diphenyl sulphide	*R_F values (× 100) for solvent**				*M.p.* °C	*Spot colour*	*Absorption max.* (*mμ*)
	A	*B*	*C*	*D*			
2-Amino-5-chloro-2′,4′-dinitro-	46	33	25	16	168	yellow	244, 320, 394
2-Amino-4-chloro-2′,4′-dinitro-	52	39	30	21	186	yellow	245, 320, 395
2-Amino-5-methyl-2′,4′-dinitro-	49	35	27	17	166	yellow	245, 320, 394

* Solvent systems: A = benzene; B = benzene–hexane, 70:30; C = benzene–hexane, 50:50; D = pyridine–hexane, 10:90.

because their spectra are quite distinct and the intense absorptions are detectable even at a very low concentration (0.0080 to 0.0165 mg/ml). All the phenothiazines exhibited two sharp peaks in the ranges of 240–255 mμ and 285–318 mμ. The sulphides exhibited a sharp peak in the visible region at 395 mμ in addition to two sharp bands in the U.V. region at 245 mμ and 320 mμ.

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CHROM. 3717

The separation of polyols by thin-layer chromatography*

Many procedures have recently been proposed for the separation of polyols by thin-layer chromatography on silica gel, alumina or Kieselguhr G plates using various developing solvents[1]. In some cases, adsorbents modified with complexing agents such as boric acid[2] or ammonium borate[3] have also been tried.

In previous work[4–6] it has been shown that in an alkaline medium complex formation takes place between lead(II) and polyols (P) containing at least two adjacent hydroxyl groups, according to the following equilibrium:

$$Pb(OH)_3^- + P \leftrightharpoons PbP^- + nH_2O.$$

Because the complex formation is affected by the number and position of the hydroxyl groups and the equilibrium constants are quite different, lead(II) has been used here as a complexing agent in the adsorbent layer to separate polyols by thin-layer chromatography. The influence of complex formation with lead(II) and of eluent composition on the mobility of polyols has been studied.

Experimental

Thin-layer plates were prepared according to the following procedure: 30 g of Silica Gel H (Merck) were mixed with 70 ml 0.1 *M* $Pb(NO_3)_2$ (Erba RP, Milan) or 70 ml water (untreated plates) by shaking in a stoppered conical flask, and were then transferred to the spreader (Chemetron, Milan). The thickness of the layer was set at 0.25 mm. All the plates were activated by heating at 110° for 30 min. Samples were applied to the starting line of the chromatograms as dilute solutions in alcohol or water–alcohol, in chromatography tanks. Alcohol–ammonia or alcohol–ammonia–water mixtures were used as developing solvents, and the polyols were detected by spraying with potassium permanganate containing sodium carbonate solution. Experiments carried out with Silica Gel G plates have not given satisfactory results. This seems to be due to the reaction of lead(II) with calcium sulfate present in the Silica Gel G.

TABLE I

FORMATION CONSTANTS WITH LEAD (II) OF POLYOLS IN ALKALINE MEDIUM

Polyol	*log K*
1,3-Propanediol	−0.20
Ethylene glycol	0.30
1,2-Propylene glycol	0.30
1,2,5-Trihydroxypentane	0.40
1,2,4-Butanetriol	0.45
Glycerol	1.15
meso-Erythritol	1.93
d-Mannitol	2.78
Dulcitol	2.90
d-Sorbitol	3.42

* Work carried out with the aid of the "Consiglio Nazionale delle Ricerche".

Results

The polyols listed in Table I were examined, their formation constants, K, with lead(II) in alkaline medium (0.1 M OH^-) having been previously determined.

Typical chromatograms of some polyols on untreated Silica Gel H and lead(II)-impregnated Silica Gel H for three developing solvent systems are reproduced in Fig. 1. The influence of lead(II) is evident: the mobility of the polyols decreases with an increase of the number of adjacent hydroxyl groups, as the formation constants between lead(II) and polyols become larger.

In Table II the R_F values measured for the polyols investigated are collected. The R_F values for glycols are slightly affected by the presence of lead(II) in the adsorbent layer, while the mobility of other polyols is considerably decreased by

TABLE II

R_F VALUES × 100 OF THE INVESTIGATED POLYOLS ON SILICA GEL H (A) AND LEAD(II)-IMPREGNATED SILICA GEL H (B)

Developing solvents: (I) ethanol saturated with gaseous ammonia; (II–VII) ethanol–conc. ammonia (32 %)–water, respectively: II (21:2:0), III (23.5:2.2:1), IV (23:2.2:2), V (21:2:2.4), VI (21:2:3.5) and VII (20:2:4).

Polyols	*II*		*III*		*IV*		*V*		*VI*		*VII*		*VIII*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1,3-Propanediol	76	68	82	75	80	78	86	79					82	82
Ethylene glycol	62	59	67	60	64	60	66	62	66	62	74	68	71	66
1,2-Propylene glycol	69	65	73	67	72	67	73	69	72	70	80	76	77	74
1,2,5-Trihydroxypentane	65	61	69	64	68	64		66	70	65	78	73	76	74
1,2,4-Butanetriol	60	53	62	60	64	60	66	62	65	62	73	68	71	67
Glycerol	48	22	53	42	50	46	56	48	57	52	62	54	63	55
meso-Erythritol	33	6	39	22	38	30	45	34	49	39	51	43	56	45
d-Mannitol	7	0	14	3	15	6	22	9	30	14	27	17	37	22
Dulcitol	4	0	12	0	14	3	21	3	29	8	24	10	35	18
d-Sorbitol	4	0	11	0	12	2	17	5	26	7	22	9	34	14

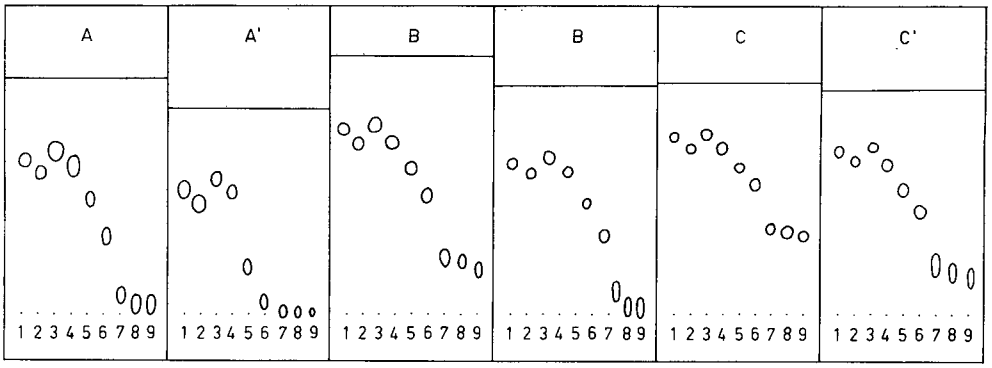

Fig. 1. Thin-layer chromatograms on Silica Gel H (A, B, C) and lead(II)-impregnated Silica Gel H (A', B', C') of the following polyols: (1) 1,2,5-trihydroxypentane; (2) 1,2,4-butanetriol; (3) 1,2-propylene glycol; (4) ethylene glycol; (5) glycerol; (6) *meso*-erythritol; (7) *d*-mannitol; (8) dulcitol; (9) *d*-sorbitol. Developing solvents: (A,A') ethanol saturated with gaseous ammonia; (B,B') ethanol–conc. ammonia (32 %)–water (23:2.2:2); (C,C') ethanol–conc. ammonia (32 %)–water (20:2:4).

the presence of the metal. A marked influence of the complexing ability of the added metal can be observed by running compounds that do not bind lead(II) on the two types of plates. Amines such as benzylamine yield identical R_F values when 95 % ethanol–25 % ammonia (4:1) is used as eluent.

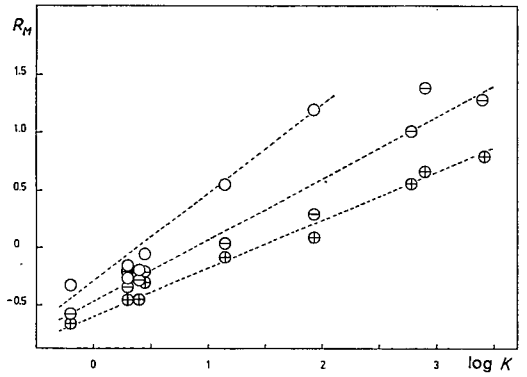

Fig. 2. R_M values plotted *versus* logarithm of the formation constants with lead(II). Developing solvents: (○) ethanol saturated with gaseous ammonia; (⊖) ethanol–conc. ammonia (32 %)–water (23:2.2:2); (⊕) ethanol–conc. ammonia–water (20:2:4).

The mobility of the polyols changes noticeably with the eluent composition. Since the polyol–lead(II) complexes are polar, their migration is affected by the polarity of the elution medium consisting of ethanol and ammonia, with and without water. The migration of glycols is slightly affected by the eluent composition. Glycerol and erythritol exhibit a large difference in the R_F values when they are chromatographed on untreated and lead(II)-treated silica gel plates when water is present in the eluent. Higher polyols show a larger difference in the R_F values on the two types of plates when the eluent does not contain water.

More favourable conditions for the separation of triols and higher polyols from diols are obtained when anhydrous ethanol saturated with ammonia is used. In this medium the higher polyols have a smaller mobility because their complexes with

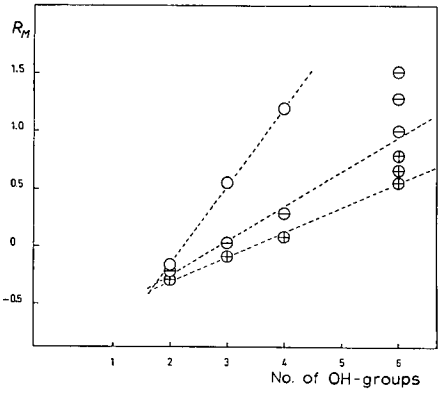

Fig. 3. R_M values plotted *versus* number of hydroxyl groups of various polyols. Developing solvents: (○) ethanol saturated with gaseous ammonia; (⊖) ethanol–conc. ammonia (32 %)–water (23:2.2:2); (⊕) ethanol–conc. ammonia–water (20:2:4).

lead(II) are stronger and very slightly soluble. It is possible to separate glycerol from erythritol and the latter from hexahydric alcohols by increasing the eluent polarity by adding water.

The dependence of the chromatographic behaviour on the formation constants of lead(II)-polyol complexes is readily observed in Fig. 2, where the R_M values obtained for three different eluents have been plotted *versus* the logarithm of the formation constants reported above. The plot is only of a qualitative nature because the formation constants were obtained under different experimental conditions (0.1 M OH^- and 1 M Na^+). It clearly shows, however, that the R_M values increase gradually as log K increases. From this plot the effect of the eluent is quite clear because the slopes of the hypothetical curves drawn through the experimental points are different.

Since R_M is a linear function of the number of equal groups responsible for the chromatographic behaviour, the R_M values obtained on lead (II)-treated plates have been plotted *versus* the number of hydroxyl groups of the polyols investigated. There is a linear relationship for diols, triols and tetrols, whereas deviations are observed for the hexahydric alcohols; this is probably due to interactions among -OH groups which influence each other.

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CHROM. 3728

Thin-layer chromatography of anthocyanins on mixed layers of polyvinylpyrrolidone and cellulose

Thin-layer chromatography (TLC) on various substrates is increasingly being used to separate anthocyanins. SOMERS[1] used cellulose TLC as a supplement to paper chromatography in separating the anthocyanins of *Vitis vinifera*, variety Shiraz. DEIBNER[2] preferred cellulose TLC to paper chromatography in separating diglucosides of malvidin and peonidin; both systems resolved the two pigments but TLC resulted in a more "distinct" separation. ASEN[3] separated anthocyanins on a preparative scale using plates layered with a mixture of silica gel and cellulose. CONRADIE AND NEETHLING[4] used silica gel TLC for separating anthocyanins of *Vitis vinifera* as did MORTON[5] for blackcurrant anthocyanins. QUARMBY[6] chromatographed anthocyanidins and other flavonoids and phenolics on polyvinylpyrrolidone (PVP) thin-layer plates. BIRKOFER *et al.*[7,8] have separated anthocyanins on polyacrylonitrile–polyamide and on alumina.

In this communication, TLC of anthocyanins on layers of PVP–cellulose mixtures is described. Incorporation of PVP in the cellulose layer resulted in more compact spots without markedly affecting the R_F value.

Experimental

Preparation of plates

Cellulose. Cellulose plates of 0.25 mm thickness were prepared by spreading a homogenate of 20 g of cellulose powder (MN-300, without binder) and 110 ml of distilled water on five 20 × 20 cm plates. The coated plates after setting at room temperature for 1–4 h were dried at 100° for 30 min and stored in a desiccator.

Cellulose–PVP. Plates of mixtures of PVP and cellulose were prepared in the same manner using the following proportions of PVP to cellulose: 1:19 (5 %), 1:9 (10 %), and 1:3 (25 %). Insoluble PVP (polyclar AT) obtained from General Aniline and Film Corp., New York, was purified as described by LOOMIS AND BATTAILE[9] to remove trace amounts of hydrogen peroxide. Particle size of 100–150 mesh was used.

PVP. 100 % PVP plates were prepared as recommended by QUARMBY[6] except that soluble PVP (Plasdone K29–32, General Aniline and Film Corp.) rather than polyvinyl alcohol was used as a binding agent.

Silica gel. Plates were prepared as described by MORTON[5].

Polyamide. The procedure of DAVÍDEK AND DAVÍDKOVÁ[10] was followed in preparing plates.

Sources of anthocyanins

Purified anthocyanin extracts of rhubarb (cyanidin-3-rutinoside, cyanidin-3-glucoside—WROLSTAD AND HEATHERBELL[11]), Bing cherries (cyanidin-3-rutinoside, cyanidin-3-glucoside—LYNN AND LUH[12]), red raspberries (cyanidin glycosides—DARAVINGAS AND CAIN[13]), strawberries (pelargonidin-3-glucoside and cyanidin-3-glucoside—ROBINSON AND ROBINSON[14] and LUKTON, CHICHESTER AND MACKINNEY[15]) and strawberry anthocyanidins (pelargonidin chloride and cyanidin chloride) were chromatographed. Anthocyanins were dissolved in 0.01 % methanolic HCl and the aglycones were isoamyl alcohol solutions. Sample size was 1 μl.

Chromatography

Plates were developed in AWHCl, glacial acetic acid–water–conc. hydrochloric acid (15:82:3), and BAW 415, less-dense phase of *n*-butanol–glacial acetic acid–water (4:1:5). Silica gel plates were also developed in BEBF, *n*-butanol–ethyl acetate–benzene–formic acid (1:1:1:1, saturated with solid paraformaldehyde), polyamide plates in 80 % methanol and 100 % PVP plates in Forestal, glacial acetic acid–water–conc. hydrochloric acid (30:10:3). Development was carried out at 21 ± 1° in the dark.

Results and discussion

In our laboratory cellulose has been used in preference to other substrates for TLC of anthocyanins. Cellulose TLC plates are not as fragile as silica gel or polyamide. Also, satisfactory separations on cellulose TLC can generally be achieved using the same solvent systems one would use for paper chromatography; this is a considerable advantage when one considers the wealth of data published on paper chromatography of anthocyanins. Paper chromatography is preferred by many for preparative work, and, since it is possible to use the same solvent systems, is very convenient.

The R_F value of some anthocyanin pigments separated on cellulose and PVP–cellulose plates with BAW 415 and AWHCl are shown in Table I. PVP affected the R_F values to a small degree in the polar solvent system while in BAW 415 the effect was more profound. Incorporation of PVP into the cellulose plate resulted in a reduction of spot size. After development in AWHCl the area of the pelargonidin-3-glucoside spot measured approximately 50 mm² on cellulose and 5 % PVP–cellulose plates and 30 mm² on 10 and 25 % PVP–cellulose plates. The smaller spot size is

TABLE I

R_F VALUES OF ANTHOCYANIN PIGMENTS SEPARATED BY CHROMATOGRAPHY ON CELLULOSE AND CELLULOSE–POLYVINYLPYRROLIDONE THIN LAYERS

Pigment	*Plate composition*	*R_F values*	
		AWHCl	*BAW 415*
Cyanidin-3-glucoside	Cellulose	0.30	0.34
	5 % PVP–cellulose	0.31	0.22
	10 % PVP–cellulose	0.29	0.20
	25 % PVP–cellulose	0.28	0.21
Cyanidin-3-rutinoside	Cellulose	0.50	0.26
	5 % PVP–cellulose	0.50	0.09
	10 % PVP–cellulose	0.47	0.11
	25 % PVP–cellulose	0.44	0.11
Pelargonidin-3-glucoside	Cellulose	0.43	0.52
	5 % PVP–cellulose	0.44	0.36
	10 % PVP–cellulose	0.42	0.28
	25 % PVP–cellulose	0.37	0.20
Pelargonidin	Cellulose	0.18	
	5 % PVP–cellulose	0.17	
	10 % PVP–cellulose	0.11	
	25 % PVP–cellulose	0.08	

particularly advantageous for two-dimensional chromatography and for densitometry work. To achieve compact spot size without markedly affecting the R_F values the 10% PVP–cellulose mixture is recommended.

The polyamide, PVP, and silica gel TLC plates were unsatisfactory with the BAW 415 and AWHCl solvent. The anthocyanins streaked severely on the polyamide plates; the 80% methanol system was more satisfactory, however, resolution was not as good as with cellulose and PVP–cellulose systems. In the silica gel–AWHCl system the pigments moved with the solvent front and in BAW 415 they were not separated. The BEBF system while not completely resolving the pigments gave a more reasonable chromatogram.

We were unsuccessful with 100% PVP plates using all three systems. The solvent front was irregular; the pigments were not separated and spread in an irregular manner during development.

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CHROM. 3718

Paper chromatography of glucose–formic acid solutions

During an investigation of the compounds found in the defensive scent fluid of the ground beetles *Dicaelus splendidus* (Say) and *D. dilatatus* (Say)[1], two-dimensional paper chromatography followed by aniline hydrogen phthalate detection revealed the presence of at least three different reducing sugars. All the spots were the same color, brown, which suggested that aldohexoses were involved but not pentoses, as the latter give reddish spots with the detecting agent used[2]. However, only one of these spots matched the R_F values of any of the listed aldohexoses, namely glucose[3].

Since formic acid was the major component in the beetles' defensive fluid, it was suspected that formate esters of glucose were present in addition to the free glucose. Several solutions of α-D-glucose in different concentrations of formic acid were prepared and chromatographed. In most cases two or three separate but sometimes hazy spots appeared, as shown in Table I.

TABLE I

R_F VALUES FOR VARIOUS GLUCOSE SOLUTIONS AT 30°

Solvents: (A) phenol saturated with water; (B) 2-butanol saturated with water; (C) 1-butanol–acetic acid–water (2:1:1, by volume).

Glucose solution	*Solvent A*	*Solvent B*	*Solvent C*
1% in water	0.47	0.25	0.43
1% in 55% formic acid	0.46	0.24	0.41
	0.67	0.38	0.53
1% in 83% formic acid	0.47	0.25	0.41
	0.66	0.39	0.52
	0.83	0.56	0.66
2% in 97% formic acid (14 months old)	0.46	0.27	0.43
	0.67	0.40	0.55
	0.85	0.58	0.67

The existence of formate esters of glucose was demonstrated by TARKOW AND STAMM[4] in a rate study which showed that esterification proceeded fairly rapidly when glucose and formic acid were mixed and that only two of the five hydroxyl groups of glucose reacted, those on positions four and six. Thus the three spots obtained from paper chromatography quite likely correspond to glucose, a glucose formate, and glucose 4,6-diformate.

These esters when spotted on paper will withstand 100° for an hour with no apparent change. However, they can be quickly and completely hydrolyzed at room temperature during paper chromatography when solvents containing NH_4OH, aliphatic amines, or HCl are used; in each instance only one spot, corresponding to free glucose, was obtained. This spot was always clear without streaking or haziness, indicating a rapid and complete conversion of the esters to free glucose. The possibility

that those solvents had been unable to separate glucose from its formate esters was excluded by two-dimensional paper chromatography. A solvent suspected of causing hydrolysis was used for the first dimension. For the second dimension, any solvent from Table I was chosen, a solvent definitely capable of separating glucose from its formate esters. Under these conditions with aniline hydrogen phthalate only one spot was detected, whose R_F values matched those of glucose. The presence of formic acid, acetic acid, or pyridine in the chromatography solvent did not cause any hydrolysis.

Lactic acid, acetic acid, and chloroacetic acid were tried as ester-forming reagents with glucose but these failed to produce any compounds different from glucose, as monitored by paper chromatography. In addition, these three acids were rather poor solvents for glucose as compared to formic acid. D-Mannose and L-rhamnose were also studied and found to react with formic acid in like manner. Mannose in 80 % formic acid yielded three spots while rhamnose, lacking a hydroxyl group on position number six, produced only two spots. Whatman No. 1 paper with ascending flow was used for all the chromatograms.

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CHROM. 3723

The separation of Alizarin Complexan from impurities by paper chromatography

Alizarin Complexan (3-[di-(carboxymethyl)aminomethyl]-1,2-dihydroxyanthraquinone) is the most important reagent for the spectrophotometric determination of fluoride[1–3]. The reagent is synthesized by Mannich condensation[4] from alizarin, iminodiacetic acid and formaldehyde in strong alkaline media[5]. The yield of the synthesis is very satisfactory, but the reagent may be contaminated by small amounts of the starting products. The different solubilities found for various samples of Alizarin Complexan confirms this opinion. Furthermore, other reagents that are synthesized

in a similar manner, *e.g.* Xylenol Orange, are also contaminated by the starting products[6].

In the present study, alizarin and iminodiacetate were separated from Alizarin Complexan; volatile formaldehyde is not expected in the final product. Paper chromatography was employed, because absorbents used for thin-layer separations may cause decomposition of products prepared by Mannich condensation[6].

Experimental

Paper. Chromatographic paper Whatman No. 1 was used for the separations.

Solvents. The following solvents were used: (S1) *n*-butanol–conc. HCl–water (8:1:1); (S2) *n*-propanol–conc. HCl–water (6:1:1).

Detection. Larger spots of alizarin are visible; smaller ones were detected by ammonia vapours, which give an intense violet colour. Iminodiacetate was detected by spraying the chromatograms with a solution containing 175 mg of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ and 10 g of KSCN in 100 ml. White spots on a red background, which turn to orange during 24 h, appear on the chromatograms.

Spots of Alizarin Complexan are readily visible. They may be detected by ammonia vapours, which give a violet colour, or by spraying with lanthanum nitrate, acetate buffer and sodium fluoride solutions according to a photometric procedure for the determination of fluoride[7].

Chromatographic procedure. About 50 mg of Alizarin Complexan are suspended in 5 ml of water and dissolved by the addition of 0.05 ml of concentrated ammonia. 0.05 ml of glacial acetic acid is then added and the solution diluted to 10 ml with water. This solution is applied on the longer side of the chromatogram (24 × 15 cm) in about 10 μl portions. The paper is rolled up to a cylinder, fastened by clips[8], and the chromatograms are developed by the ascending technique until the front has travelled 10 cm (about 2 h). Alizarin and iminodiacetate are chromatographed in the same manner. The chromatograms are dried under an infrared lamp and treated with the detection reagents.

Results and discussion

Solvent S1 (*n*-butanol–conc. HCl–water (8:1:1) proved to be the best of the various solvents examined. When the content of *n*-butanol was changed significantly, very diffuse and elongated spots were obtained. Changes in the content of hydrochloric acid had no influence on the separation. Substitution of HCl for acetic acid, which may lead to better separation of organic reagents from starting substances[6], resulted in double spots of iminodiacetate, which interfered with the spots of Alizarin Complexan. On examining other organic solvents, an excellent mixture for the separation of alizarin from Alizarin Complexan was found, *viz.* solvent S2 (*n*-propanol–conc. HCl–water, 6:1:1). Very sharp spots of alizarin at the front of the mobile phase are obtained. The R_F values for both solvents mentioned are given in Table I.

Solvent S1 may be recommended for the preparation of highly pure Alizarin Complexan, *e.g.* by column cellulose chromatography. This solvent may also be used for the determination of iminodiacetate in samples of Alizarin Complexan. For the determination of alizarin in this reagent, solvent S2 may be employed. We have determined alizarin and iminodiacetate in two samples of Alizarin Complexan. The determination was carried out by comparing spots of standards with spots of both

TABLE I

R_F VALUES OF ALIZARIN COMPLEXAN AND ITS IMPURITIES

No. of the spot	*R_F value*		*Substance*
	S1	*S2*	
1	0.18	0.37	iminodiacetate
2	0.39	0.52	Alizarin Complexan
3	0.98	1.00	alizarin

TABLE II

THE DETERMINATION OF ALIZARIN AND IMINODIACETATE IN ALIZARIN COMPLEXAN

Samples: (I) Hopkin & Williams, Ltd., Great Britain; (II) Siegfried S.A., Zofingue, Switzerland.

Substance determined	*Detection limit*	*Content in the sample* (%)	
	(μg)	*I*	*II*
Alizarin	0.1	1.6	4.2
Iminodiacetate	0.4[a]	3.8	0

[a] For an orange colour on a red background.

substances obtained in the separation of samples. The results are given in Table II, which also includes the detection limits for both substances. The results indicate a new way of washing Alizarin Complexan after it has been synthesized; the sample with a higher content of alizarin contains less iminodiacetate and *vice versa*. The determinations of alizarin and iminodiacetate in freshly prepared and 4-month-old solutions of Alizarin Complexan showed that the content of alizarin increased by 45%, while that of iminodiacetate only increased by 20% during this period. This indicates that solutions of Alizarin Complexan may be stored much longer than was previously[2] supposed. The recently observed high stability of lanthanum–Alizarin Complexan reagent in acetone–water media[7] may be due to the stability of the solutions of Alizarin Complexan.

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CHROM. 3722

Accelerated analysis of some amines and amino acids

Quantitative analyses for *p*-tyramine, cadaverine, putrescine, histamine, and tryptamine were found to be necessary in the course of some studies of the amino acid metabolism of grass silage on an automatic amino acid analyzer. Estimations of these silage components have been carried out by quantitative paper chromatography[1–3], but a method which would require the minimum of desalting or other pretreatment was considered to be desirable.

The studies of PERRY AND SCHROEDER[4] on the range of amines found in urine samples indicated that an extension of automatic amino acid analysis[5] to amine analysis was possible if weak (carboxylic acid) cation exchangers were used in place of the strong (sulphonic acid) cation exchange resins in the chromatographic columns.

Methods and materials

Samples of the polymethacrylic acid exchanger used by PERRY AND SCHROEDER[4], Amberlite CG-50 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), and the polyacrylic acid exchanger, Zeo Karb 226 (2.5 % and 4.5 % DVB grades, Permutit Co., Ltd., London), were fractionated in the sodium salt form by the method of HAMILTON[6]. Those particles which were washed through a 2 l separating funnel at flow rates between 50 and 200 ml/min were found to be most suitable for the chromatographic studies described in this paper.

Sample volumes were 0.5 ml or less and were loaded on the columns by application of gas pressure; the inside of the chromatographic tube was carefully washed with three 0.2 ml portions of the eluting buffer before the column was filled with buffer to begin an analytical run.

The pyridine–acetic acid buffers used by PERRY AND SCHROEDER[4] were found to swell the resins and soften them in such a way as to prevent adequate flow rates to be used with the desired particle sizes. Since the purpose of the separations was purely analytical, there was no objection to inorganic buffer salts, and the method was accordingly developed with sodium and potassium salt buffer systems.

Amberlite CG-50 resins are only available as ground materials in the particle sizes used, but these irregular particles were found to be too fragile to allow a fixed bead size to be maintained in use in small columns. The bead form resin, Zeo Karb 226×4.5 (Permutit SRC-48, batch 4) was the only commercially available product which could be made to give adequate separating power with reproducible hydraulic properties.

Basic amino acids. It was soon obvious that the weak cation exchange columns were just as efficient for separations of basic amino acids as the conventional stronger acid exchangers, with the proviso that very weakly basic amino acids such as 4-aminobutanoic acid and 3-aminopropanoic acid were not differentiated from the neutral and acidic amino acids. Table I gives the elution times of the (completely separated) basic amino acids on a column (14 by 0.5 cm) of Zeo Karb 226×4.5 (20 ± 8 μ bead diameter) at a flow rate of 31 ml/h of sodium maleate (0.15 *M*) buffer (pH 6.83) at 48°.

Amines. Unfortunately, silage extracts contain significant quantities of 4-amino-

TABLE I

ELUTION TIMES OF THE BASIC AMINO ACIDS

Amino acid	*Elution time (min)*
Lysine	25
Ornithine	32
Histidine	38
Arginine	46
Ammonia	55

butanoic acid, so further development of the weak acid resin columns was limited to the amine components alone. Amberlite CG-50 was the most efficient resin tried for the separation and at 48° complete resolution of all the amines present in an extract was readily achieved on a column (16 by 0.5 cm) of CG-50 (average particle diameter 20 μ) at a flow rate of 31 ml/h of sodium maleate buffer (pH 6.14, 0.12 *M* disodium maleate plus 0.18 *M* sodium chloride, 1.04 *M* in sodium) in rather less than 2 h, but the continuous fragmentation already mentioned prevented use of this granular resin. The Zeo Karb 226 × 2.5 resin was also very efficient, but it was so soft that even moderate operating pressures of 50 p.s.i. caused bead deformation and consequent rapid increase of the operating pressure.

TABLE II

ELUTION TIMES OF THE PHYSIOLOGICAL AMINES

Amine	*Elution time (min)*
Ammonia	31
2-Amino-3-guanidino-propanoic acid	49
p-Tyramine	81
Cadaverine	95
Putrescine	104
Histamine	140
Tryptamine	165

Satisfactory results were obtained for amine analyses with the Zeo Karb 226 × 4.5 exchanger only after a switch from sodium to potassium buffer salts. Table II gives the elution times of the amines (and the 2-amino-3-guanidinopropanoic acid used as internal standard) on a column (22 by 0.5 cm) of this latter resin (20 ± 8 μ bead diameter) at 52°, and a flow rate of 28.5 ml/h of potassium citrate buffer (pH 6.15, 1.1 *M* in potassium, composition as given in Table III). Back pressure with this system is about 80 p.s.i. and the column can be used (without regeneration) for at least a dozen analyses before deterioration of the resolving power becomes noticeable.

The separations were monitored by continuous colorimetry using a standard Technicon (Technicon Instruments Co. Ltd., Chertsey, Surrey, England) amino acid analytical system with 1.5 and 0.8 cm path length flow cuvettes. With this system

TABLE III

POTASSIUM CITRATE BUFFER FOR AMINE SEPARATION

Component[a]	*Amount*
Potassium concentration (*M*)	1.10
Citrate concentration (*M*)	0.033
Potassium citrate (g)	21.6
Potassium chloride (g)	149.1
Brij 35 (ml) (50 g/100 ml)	20
Octanoic acid (ml)	0.20
Final volume (l)	2
Concentrated hydrochloric acid	to pH 6.15 ± 0.02

[a] All reagents except Brij 35 are "Analar" grade from British Drug Houses Ltd., Poole, Dorset, England. Brij 35 (polyoxyethylene lauryl ether) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

the diamines, cadaverine and putrescine, gave ninhydrin colour yields equivalent to those of their parent amino acids, lysine and ornithine; the three monoamines, *p*-tyramine, histamine, and tryptamine, were slightly less reactive and gave about two-thirds of the colour yield of lysine. The complete analytical system allows reasonably rapid determination of samples containing 0.25 to 0.01 μmoles of each amine component with a reproducibility of ± 3 %.

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Book Reviews

Electrophoresis: Theory, Methods and Applications, Vol. II, edited by MILAN BIER, Academic Press, New York and London, 1967, XVIII + 553 pp., price $ 21.

Since publication in 1959 of the first volume of BIER's Electrophoresis, progress in electrophoretic methodology and the significance of its contribution to elucidation of the molecular mechanisms of life processes have been extraordinary. Like ultracentrifugation, gel filtration and chromatography, electrophoresis is playing an indispensable role in the development of many areas of biology and medicine. During this period, theoretical advances have also been made in our understanding of the factors governing the rate of electromigration and the electrophoretic transport of interacting systems. Many of these developments are discussed in Vol. II. Thus, for example, the first chapter, written by J. TH. G. OVERBEEK AND P. H. WIERSEMA, is a logical and critical extension of Chapter 1 of Vol. I. The latter, by J. TH. G. OVERBEEK AND J. LYKLEMA, was a theoretical treatment of the electrical double layer surrounding charged particles in solution and its relationship to electrophoresis. In Vol. II, the authors address themselves to the important problem of evaluating the ξ-potential from experimental values of the electrophoretic mobility, μ. Particular emphasis is placed upon the contribution of the *relaxation effect* to the forces acting on a hard impermeable sphere in an electric field. The authors indicate how one can decide whether Henry's equation is appropriate for computation of ξ from μ. If it is not, recourse can be had to recent numerical solutions of a set of theoretical equations which do include the relaxation effect. The more difficult problems of interpreting the mobilities of nonspherical particles and random coil polyelectrolytes is also considered. But, this is virtually the full theoretical coverage of the book. It is unfortunate that, while mention is made on p. 414 of the fact that multiple electrophoretic zones need not necessarily indicate inherent heterogeneity but may arise from several different types of interactions of a single macromolecule, the editor chose not to devote a section to the theory of electrophoresis and ultracentrifugation of reversibly reacting systems. In addition to its importance for the detection and characterization of biologically important macromolecular interactions, such theory is providing the understanding required for unambiguous interpretation of the more conventional analytical applications of moving-boundary and zone electrophoresis.

The principles and applications of starch and polyacrylamide gel electrophoresis, automatic density gradient electrophoresis and electrophoresis in semifluid films are discussed in three chapters written by H. BLOEMENDAL; H. HOCHSTRASSER, H. LERNER AND L. T. S. SKEGGS, JR.; and N. RESSLER. One is impressed not only by the high resolving power of these methods but also by the sophisticated spectroscopic methods now available for recording zone patterns. Description of the several techniques for isolating macromolecules from their separated zones is welcomed, since isolation and electrophoretic analysis of fractions provides an unambiguous means for distinguishing between inherent heterogeneity and interactions.

An interesting although somewhat incomplete chapter by K. HANNIG is concerned with several recent advances in preparative electrophoresis and its applications. Forced-flow electrophoresis and continuous zone electrophoresis in a free film of buffer are described in detail, but no mention is made of continuous density gradient electrophoresis as practiced by MEL (see, however, Chapter 10 for literature citations). With these developments electrophoresis has evolved to the point where it is now possible to separate mixtures representing the whole size-spectrum of charged particles—low molecular weight inorganic and organic substances, proteins, viruses, bacteria, mammalian cells or cell organelles.

Finally, six chapters are devoted to the applications of electrophoresis to problems of identification, structure and genetic variability of proteins and the composition of complex biological tissues and fluids. These are written by F. SŐRM AND B. MELOUN; P. BURTON AND P. GRABAR; B. H. BOWMAN; H. M. GREY; F. C. COURTICE; and G. B. J. GLASS. While these chapters illustrate the powers of electrophoresis in biology, the reviewer found some sections tedious. In fact, the lengthy description in Chapter 2 of methods of sequencing peptides and proteins seems irrelevant to this book. Moreover, the organization of the book is curious. Rather than progressing from theory to methods to applications, the six chapters on applications separate the first, theoretical chapter from the final four chapters on analytical and preparative methods. Nevertheless, the book should enjoy a wide readership including clinical chemists, biological and medical investigators, and those concerned with applications of electrophoresis to commercial production of highly purified biological materials and to large-scale water purification.

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Analytical methods for pesticides, plant growth regulators and food additives, Vol. V, edited by GUNTER ZWEIG, Academic Press, New York, 1967, 570 pp., price $ 24.

This series was originally planned in four volumes, but with new pesticides coming forward at a steady rate and new methods of analysis being developed, at least one supplementary volume was inevitable. Volumes I–IV constitute a valuable consolidation of knowledge in the field of pesticide analysis (especially since much of the information was previously held in commercial files, and had not been published) and is now a basic reference work in most pesticide laboratories.

Volume V follows the pattern already set and is, in general, well-planned and well-executed. The authors, all American, each an expert in his own field, have achieved a substantially uniform and fairly high standard of presentation. It seems a pity though, to a reviewer on this side of the Atlantic, that the chapters on diquat and paraquat could not have been written by an analyst from Plant Protection Limited who developed the analytical methods. Like the other four volumes there is a strong bias to American practice and this is most apparent in the uneven use of nomenclature.

It would be helpful if the authors used common names when available, in preference to trade names. "Tordon" and "Banol" are registered trade names, but are not indicated as such in the contents list.

The first three chapters on newer analytical methods are useful compilations, but contain little more than recent reviews on the subjects. The chapter on polarographic methods is nevertheless a welcome recognition of this very useful and often under-rated technique. On page 78, however, a method for biphenyl in synthetic fatty acids is considered under the heading of organometallic pesticides. More careful editing could have removed this anomoly.

By far the most interesting chapters are those dealing with general applications of residue methods to the analysis of Water Resources, Fish and Wildlife and the chapter on Principles of Pesticide Metabolism Studies, but unfortunately many valuable non-American papers on wildlife analysis are not referred to. The chapter on Water Resources contains several phrases which draw comment, for example, on page 84 "certain insecticides having an acute toxicity, for example toxaphene..." The classification of pesticides on page 86 can best be described as quaint and could certainly be improved.

The remaining 32 Chapters are monographs on individual pesticides, most of which have achieved commercial importance in the United States since 1963. The evaluation of these monographs is difficult since methods became out-of-date so quickly as analytical techniques are developed. For example, most laboratories now determine residues of dinitrophenyl compounds by thin-layer chromatography and/or polarography whereas the chapter on DNBP recommends an older colorimetric technique. The chapters too are very uneven in the data provided on the reproducibility, accuracy and the limits of determination of the residue methods. These are most important to an analyst considering any method.

In spite of a certain number of small errors, this book is again most welcome, and taking into account the current developments in pesticide analysis it seems almost certain that a further volume in this series will be forthcoming. One important aspect of residue analysis so far missing from this series is a chapter on the evaluation of results. The value of much useful analytical work is reduced by errors in interpretation of results, and a chapter on this aspect would serve a very useful purpose.

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Gas Chromatographic Analysis of Drugs and Pesticides, by BENJAMIN J. GUDZINOWICZ, Marcel Dekker Inc., New York, and Edward Arnold, London, 1967, ix + 605 pp., price $ 28.50.

This book is written in two parts, of which part I is a general introduction to gas chromatography and part II is a review of the application of gas chromatography to the analysis of drugs and pesticides.

Part I consists of three chapters devoted to (1) History and general theory of gas chromatography, (2) Detectors and their principles of operation and (3) Use of gas chromatographic data in qualitative and quantitative analysis. The material is all the good, solid stuff with which gas chromatographers are now familiar. The author has, perhaps, included material which is unlikely to be helpful to the analysts at whom the book is aimed, *e.g.* the mathematical treatment in chapter 1 is more advanced than is necessary in an analyst's handbook, while it falls short of what would be required in a theoretical textbook. The discussion in chapter 3 on the correlation of retention data with such physical properties as boiling point, density, viscosity, parachor etc. is most interesting to a physical chemist but not very relevant to the identification of a substance unless at least 100 ml of pure sample is available! I have reservations, too, about the advice on p. 23 to re-sieve the column packing material after impregnation with the liquid phase; I have always preferred to handle the material as little as possible at this stage to minimise the risk of particles breaking up and exposing uncoated surfaces.

Part II contains the following chapters: (4) Phenothiazine drugs and barbiturates; (5) Phenylethylamine type and tryptamine-indole base alkaloids; (6) Morphine, nicotine and pyrrolizidine-related alkaloids and marihuana cannabinols; (7) Antihistamines, high-boiling amine anaesthetics and vitamins; (8) Miscellaneous drugs and pharmaceuticals; (9) Pesticides, herbicides and related compounds. Each chapter consists of a review of published work on the detection, identification and determination of the chemicals concerned. Both assay and determination of drugs in body fluids are covered in many cases in chapters 4–8; in the case of pesticides the emphasis is on crop residues determination. The text includes many chromatograms indicating the resolution and separation obtainable and many tables of retention data. The usefulness of a few of the latter is, unfortunately, impaired by the omission of such details as the stationary phase used or the column temperature; the table on p. 207 is an example in which the information is not even extractable from the text. On the whole, however, the tables of retention data should be a most useful part of the book. Various techniques to facilitate identification are described such as the use of specific and non-specific detectors, gas chromatography of pyrolysis products and temperature programming.

One of the minor irritations of this book—and of American textbooks, generally—is the indiscriminate use of "approved" coined names and proprietary names to refer to drugs and pesticides; often proprietary names are used even where an "approved" name also exists, as for example "Guthion" for azinphosmethyl and "Staphcillin" for methicillin.

The purchaser of this book will obtain a comprehensive review—descriptive rather than critical—of gas chromatographic techniques which have proved useful in the drugs and pesticides fields; he should realise, however, that the author has

confirmed his attention to the gas chromatography stage of the analysis and offers no advice on the preliminary clean-up of biological samples for drug determination and only abstracts of methods of clean-up for pesticide residues work. However, the book has a complete bibliography at the end of each chapter and the reader should have little difficulty in tracking down the experimental details of the methods referred to. The analyst about to make a start on the use of gas chromatography for drugs and pesticides analysis would be well advised to purchase this book; if he is also a newcomer to gas chromatography, part I should give enough background information to make effective use of part II.

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Lipid Chromatographic Analysis, Vol. I, edited by G. V. MARINETTI, Edward Arnold, London, and Marcel Dekker, New York, 1967, 537 pp., price £ 10.10.0.

This volume consists of separate articles by leading workers in the lipid separation field and covers paper, thin-layer and column chromatography of phosphatides, glycolipids, neutral glycerides, fatty acids, and partial hydrolysis products of phosphatides and glycolipids. Gas chromatographic separation of neutral glycerides, alkoxy lipids, fatty acids, aldehydes, sphingosides and of compounds such as inositol, glycerol, carbohydrates, and nitrogenous bases derived from lipids is also described.

As might be expected the sections vary in quality from authorative expositions to somewhat disappointing incomplete coverage. It is perhaps a pity that at this advanced stage in the development of lipid chemistry and biochemistry sections have been based on techniques. A more useful basis would be the different classes of compound. No laboratory uses only one technique and in any case the thin-layer plate, the liquid column and gas–liquid chromatography are not exclusive but complementary. Anyone entering the field would be somewhat baffled at the alternative methods described in widely spaced chapters.

Nevertheless this book is a useful compendium of data on the separation techniques used for lipids, even though the price is rather high.

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News

Manufacturers' literature

HCL Scientific, Inc., a new company in the field of gas chromatography, has acquired the *Warner-Chilcott* (Research-Specialties) 1600 *Series Gas Chromatography Line* from American Optical Instrument Co., effective March 1st, 1968.

The HCL Scientific 1600 Series is a completely modular instrument for glass, metal or capillary columns using thermal conductivity, flame ionization and electron capture detection systems. The line has had very good acceptance in the biomedical field, both in research and clinical laboratories.

HCL Scientific will market and manufacture a full line of GC equipment, accessories and supplies. HCL will also provide field and factory repair service for all Warner-Chilcott and Research Specialities gas chromatographs now in use.

In addition to the home office and manufacturing facilities located in Rockford, Ill., HCL has established a regional sales office in Hamden, Conn.

For further information apply to the publisher under reference No. Chrom. N-139.

Perkin-Elmer Ltd., Beaconsfield, Bucks., have released details of their Model 270 *analytical mass spectrometer* incorporating a modified Model 900 gas chromatograph as an integral part of the analyzer allowing the use of packed, capillary and support coated open tubular columns.

For further information apply to the publisher under reference No. Chrom. N-154.

Chemapol, Prague, Czechoslovakia, have distributed a leaflet written by T. I. PŘISTOUPIL of the Institute of Hematology and Blood Transfusion, Prague, describing the use of *Synpor membrane filters* in the use of nitrocellulose membranes for the microchromatography and microelectrophoresis of proteins and other substances.

For further information apply to the publisher under reference No. Chrom. N-149.

Research Notes, February 1968, from Varian Aerograph contains articles on:
(i) The use of gas chromatography in solving the recent tragedy in Tijuana, Mexico.
(ii) Computers and gas chromatography.
(iii) Collecting GLC effluents with cigarette filter tips.
(iv) How accurate are digital integrators?
(v) Capillary column inlet splitters.

For further information apply to the publisher under reference No. Chrom. N-145.

Apparatus

New literature on the *Series RD single pen strip chart recorder/controller* is available from Barber-Colman, Rockford, Ill.

The Series RD instrument is only 8–7/8 in. wide and 10–1/2 in. high, yet makes use of a full 6–1/2 in. scale and chart. It is a null balance potentiometric instrument with solid state circuitry. The measuring circuit is fully shielded and guarded. Common mode and series mode rejection is high. When the instrument is used with an intermediate proportional controller, a 100 % control slidewire is provided. Up to four front set and two back set control switches can be supplied.

For further information apply to the publisher under reference No. Chrom. N-141.

Waters' new *Analytical Chromatographic Developer* (ACD-100)—introduced at the 1968 Pittsburgh Conference in Cleveland—is designed to automatically equilibriate, develop and dry thin-layer plates and paper strips under controlled conditions. Since each step in the process is automatically programmed to maintain analytical conditions at optimum levels, more reproducible thin-layer chromatographic (TLC) separations are obtainable with the ACD-100 than with most manual methods now in use. In this way, certain inherent disadvantages of other TLC techniques, which may adversely affect the reliability of the results, are minimized or completely eliminated. This is particularly important in biological and pharmaceutical studies in which TLC is used to investigate a wide range of such materials.

For further information apply to the publisher under reference No. Chrom. N-156.

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Gas Chromatography

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33b. Volatile inorganic substances

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35b. Antioxidants and preservatives

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Errata

J. Chromatog., 35 (1968) 389–395.

Page 394, 2nd line after Table II, "5-γ quantities" should read "1-γ quantities".

J. Chromatog., 36 (1968) 253–258.

Page 255, Table II. Under the heading *Unsaturated derivatives* "Δ^6" should read "Δ^5"

Page 257, line 9 of the Experimental section, "No. 14 paper" should read "No. 4 paper".

J. Chromatog., 36 (1968) 381–383.

Page 381, line 11 of the Experimental section, "50 μg/ml" should read "50 mg/ml".