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QUANTITATIVE ASPECTS OF PYROLYSIS/GAS–LIQUID CHROMATOGRAPHY OF SOME VINYL POLYMERS

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SUMMARY

Using a filament type pyrolyser and a specially constructed control unit, the depolymerisation behaviour of acrylates, methacrylates and styrene homopolymers and copolymers was examined by "stepwise" and "one shot" pyrolysis under controlled conditions.

The possibility of pyrolysis/gas–liquid chromatography as a technique for distinguishing mixtures of homopolymers from copolymers as well as for quantitative measurements is shown.

Some theoretical considerations of thermal degradation of vinyl polymers are also given.

INTRODUCTION

From the mass of information on thermal degradation of vinyl polymers[1,4–8,10–15] it may be concluded that such polymers have an optimum depolymerisation temperature at which a maximum monomer yield is obtained. Above this temperature the monomer yield decreases, with the formation of secondary and higher breakdown products. An example of this is in the work of MADORSKY AND STRAUS[1], who examined the breakdown products of polystyrene between 360° and 850° by mass spectrometry and found that at 360° 94.4 % of the volatile products consisted of monomer and 5.6 % toluene, whereas at 850° the volatile products contained 34 % monomer, 58.2 % benzene, 5.5 % toluene and 1.9 % propadiene. Generally this behaviour was confirmed by LEHMANN AND BRAUER[2], who examined the depolymerisation products by pyrolysis/gas–liquid chromatography.

It appears, therefore, to be a justifiable assumption that if a high monomer yield and avoidance of the formation of secondary and higher breakdown products is sought it would be advisable to carry out depolymerisation within a certain temperature range.

The primary aim of this work was to establish such depolymerisation conditions and investigate the quantitative aspects of the behaviour of acrylic polymers and copolymers on depolymerisation. In the course of this work, however, it was found that such polymers have characteristic depolymerisation patterns which make it

possible to distinguish between mixtures of homopolymers and copolymers. Further work on polymers of known composition would undoubtedly be necessary in order to elucidate certain breakdown behaviour, but the aim of this paper is to illustrate the practical usefulness of the technique used.

EXPERIMENTAL

Apparatus

The pyrolyser. This is a coil made from 9 cm of 30 S.W.G. nichrome wire wound on a No. 6 wood screw and spot welded to tungsten leads into a B7 cone (Fig. 1a). The hollow part of the cone is filled with an aqueous solution of sodium silicate and allowed to solidify. The cone is placed into the inlet of a Perkin-Elmer F11 gas chromatograph provided with a B7 socket (Fig. 1b).

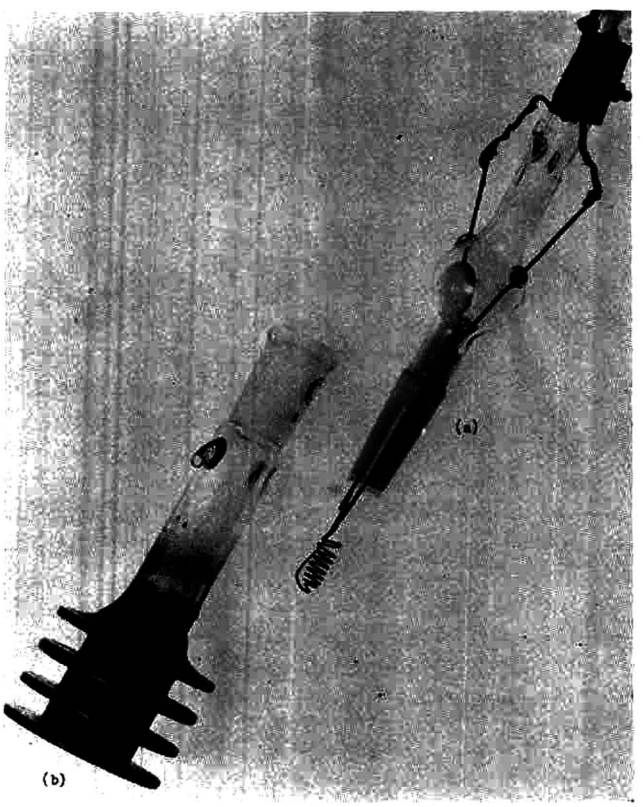


Fig. 1. The pyrolyser used in this work.

The pyrolyser control unit. This unit is basically that described by BARLOW, LEHRLE AND ROBB[3] in which the selector switch giving coil temperature increments of 100° between 150° and 950° was replaced by a variable resistance (Variac) allowing pyrolysis to be made at any desired temperature. The unit also controls a booster which brings the initial temperature of the coil to just below the breakdown tempera-

ture of the polymer and may also be used to evaporate any residual solvent prior to depolymerisation. Provisions for the control of the length of time of the electrical impulse ("flashing time") and burning off the polymer residues at a high temperature are also incorporated.

Once the desired conditions are set, the depolymerisation sequence is performed automatically by pressing the push-button marked "prime" on the top left side of the depolymerisation unit (Fig. 2).

The gas chromatograph used was a Perkin-Elmer F11 with temperature programming and a flame ionisation detector. The inner tube carrying the gas to the column was extended so as to be approximately 2 mm from the end of the coil.

A 2 m column, packed with 20% polyester (DEGS) on Celite (60–80 mesh) was used throughout this work except in the case of methyl methacrylate/ethyl acrylate copolymers when a 4 m 20% silicone oil column was used.

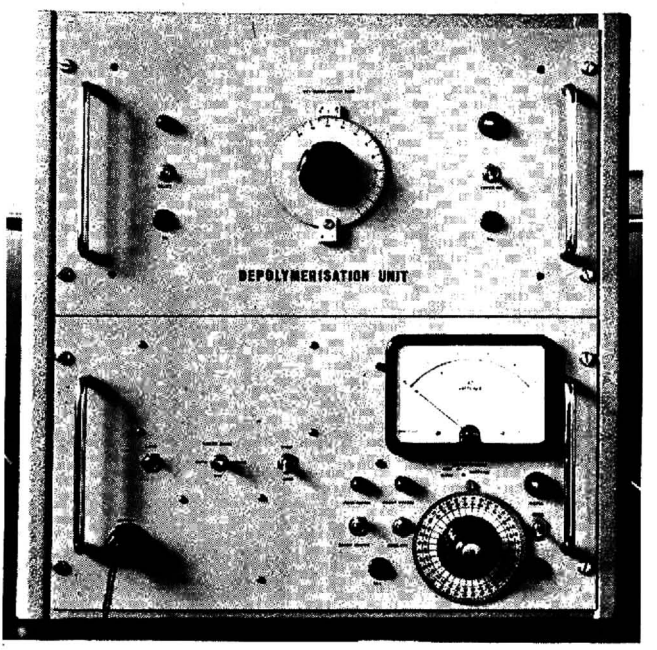


Fig. 2. The pyrolyser control unit.

Depolymerisation conditions

Depolymerisation temperature. The average temperature of the coil at any setting of the "Variac" was found by calibrating with compounds of known melting points. The temperature, however, varies along the length of the helix and in order to obtain reproducibility, the sample, in solution, was always placed on the smallest turn of the helix. Although this ensured reproducibility, the temperature should not be regarded as the true temperature.

Flashing time. The influence of the length of time of the electrical impulse (flashing time) was investigated using methyl methacrylate, styrene and ethyl

acrylate homopolymers. In each case when the same weights of the samples (10 μg) were depolymerised under individually identical conditions but with flashing times of 10, 20 or 30 sec, no differences were observed in the shapes or heights of the peaks of the individual monomers evolved. It was, therefore, concluded that, for this weight, the depolymerisation took place within the first 10 sec of flashing and this time was used in all subsequent work.

Sample weight. It is reasonable to assume that the smaller the sample the more efficient the depolymerisation.

The effect of the sample weight on the monomer yield of polymethyl methacrylate is shown in Table I. Samples of 1, 2, 3, 4 and 5 μl of a solution containing 2.52 μg/μl of polymethyl methacrylate in benzene were depolymerised at the optimum temperature in duplicate.

TABLE I

Weight (μg) depolymerised	*% of monomer yielded*
2.52	100* ± 1.0
5.04	95.0 ± 1.1
7.56	88.5 ± 0.0
10.08	83.5 ± 2.5
12.60	73.0 ± 1.1

* Assumed 100%.

Thus in the case of polymethyl methacrylate the monomer yield decreases with increasing sample size.

In the case of styrene and ethyl acrylate homopolymers, the monomer yield of samples varying in weight between 4.5 and 22.5 μg and 5–25 μg respectively increased steadily with weight within the ranges tested.

Generally in this work, the sample size varied between 5–10 μg. In the case of acrylates, however, because the monomer yield is low, larger sample size was required.

Carrier gas flow rate. Nitrogen was used as carrier gas and controlled by a Perkin-Elmer flow control unit at 25 ml/min.

Depolymerisation procedure

In order to find the optimum depolymerisation temperature samples were depolymerised in steps at increasing temperatures and the monomers evolved recorded. Two procedures were followed:

(i) In the first a known volume of polymer solution of known concentration was placed in the smallest turn of the helix using a 10 μl Hamilton syringe. The helix was placed in the B7 socket of the chromatograph and the booster switched on in order to flash off the solvent. When the chromatograph reached stable conditions, the sample was depolymerised at 250° and the monomer evolved recorded. The coil was then removed and cleaned by burning off the residue. It was then recharged and the same procedure repeated at 25° increments.

(ii) Because this procedure was too lengthy, a second procedure of stepwise depolymerisation was tried. In this procedure the residue from the pyrolysis at a lower temperature was used for the next step until complete depolymerisation was

achieved. The stepwise depolymerisation of polymethyl methacrylate by the two techniques is illustrated in Fig. 3. The traces show the peak areas of the monomers evolved plotted against temperature. Fig. 3a, which is the first type of depolymerisation, shows a maximum monomer evolution at 410° after which the monomer yield decreases with temperature, while the stepwise depolymerisation, Fig. 3b, appears similar to the first derivative of the first procedure. This second procedure was used in all subsequent work.

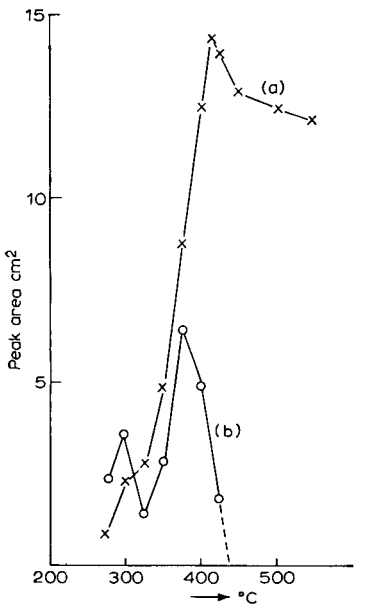


Fig. 3. (a) Constant weight and (b) stepwise depolymerisation of polymethyl methacrylate.

RESULTS

Distinction between copolymers and homopolymer mixtures

Applying the stepwise depolymerisation procedure to various polymers it was found possible, because of the differences in the depolymerisation temperature, to distinguish copolymers from mixtures of homopolymers (Figs. 4–6).

The stepwise depolymerisation procedure is thus a useful method for distinguishing mixtures of homopolymers from copolymers. Once this is established, the unknown polymer may, thereafter, be depolymerised at one temperature (its optimum temperature) by 'one shot' depolymerisation. The two procedures, however, do not give identical yields. Some results obtained by the two techniques are given in Table II and will be discussed later in considering the mechanism of depolymerisation.

Monomer yield of copolymers on one shot depolymerisation

The monomer yield obtained from homopolymers is not repeated in their copolymers. Indeed the trend in Table II is reversed and higher yields of acrylates

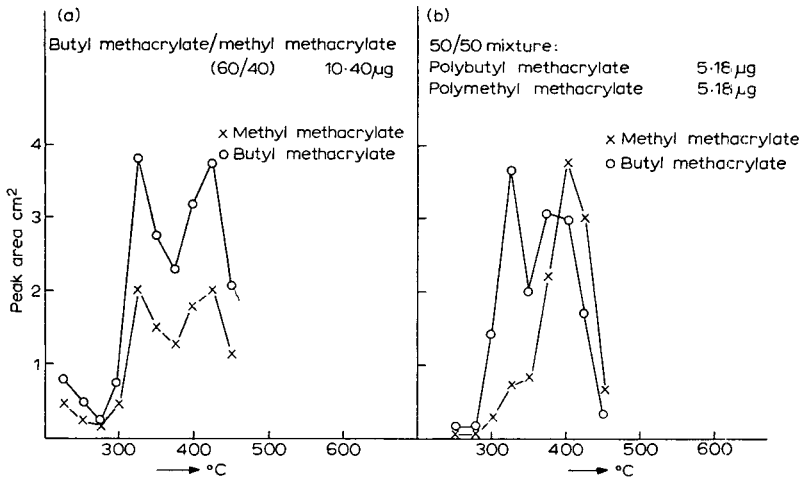


Fig. 4. Stepwise depolymerisation of (a) polybutyl methacrylate/methyl methacrylate copolymer and (b) a mixture of homopolymers.

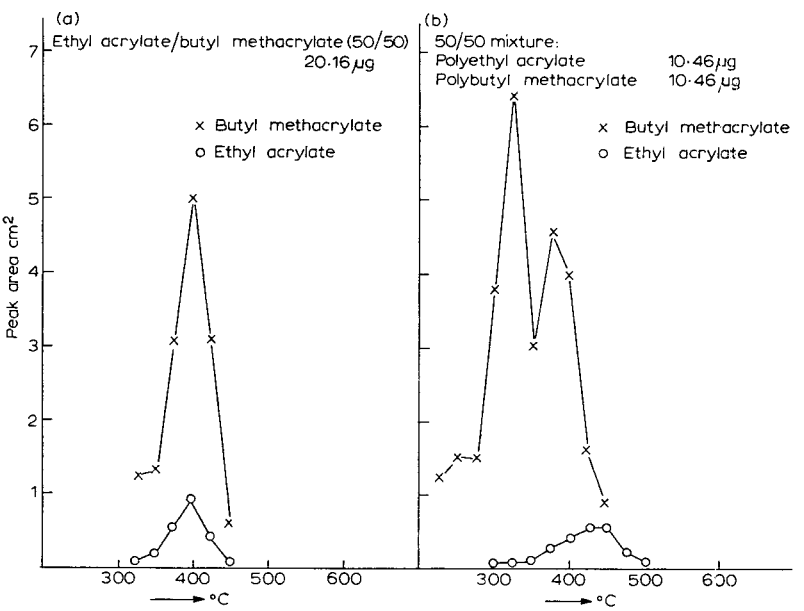


Fig. 5. Stepwise depolymerisation of (a) polyethyl acrylate/butyl methacrylate copolymer and (b) a mixture of homopolymers.

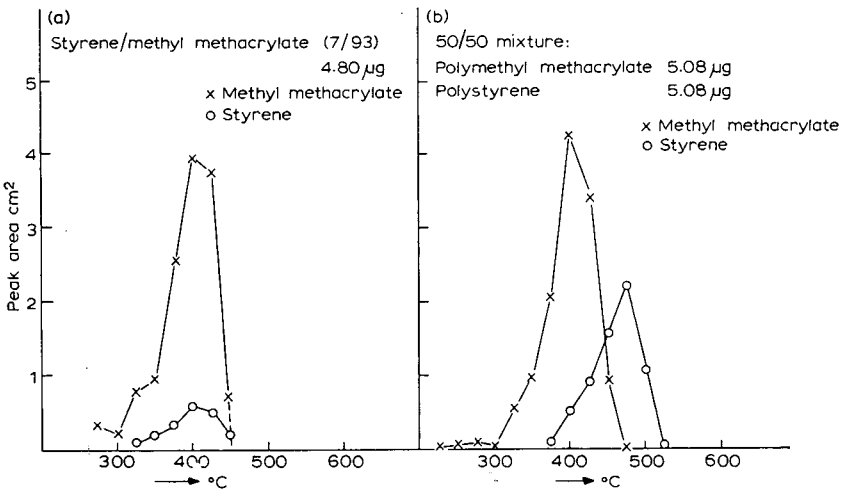


Fig. 6. Stepwise depolymerisation of (a) polystyrene/methyl methacrylate copolymer and (b) a mixture of homopolymers.

TABLE II

Polymer	*% monomer yield*	
	One shot	*Stepwise*[b]
Polymethyl methacrylate, mol.wt. 35,000	49.5	63.3
Polymethyl methacrylate, mol. wt. 216,000	50.0	74.7
Polybutyl methacrylate[a]	95.0	95.0
Polyethyl acrylate[a]	7.0	6.6
Poly-2-ethylhexyl acrylate[a]	12.0	2.5
Polystyrene[a]	48.6	49.0

[a] Molecular weight unknown.
[b] Summed total.

and lower yields of styrene and methacrylates are obtained from copolymers as shown in Table III.

Since the monomer yield varies so greatly with the type of polymer, one would assume at first glance that quantitative estimation would be impossible. However, a linear relationship can be shown to exist between the ratio of the monomers in the polymer and the ratio of the peak areas of the monomers evolved as is shown in Table IV and illustrated in Figs. 7, 8 and 9.

Thus quantitative analysis is possible provided it is first ascertained that the material is a copolymer and not a mixture of homopolymers. The extension of this conclusion to other copolymers, multi-component polymers and block copolymers has not been checked, but it seems reasonable to assume that block copolymers would behave like a mixture of homopolymers. In preparing calibration graphs of multi-component polymers, in order to provide valid quantitative estimation, only one of the components should be varied at a time.

TABLE III

ONE-SHOT DEPOLYMERISATION OF A SERIES OF HOMOPOLYMERS AND COPOLYMERS AT 500°

Polymers	*% monomer yield (on polymer)*	
	Ethyl acrylate	*Methyl methacrylate*
Methyl methacrylate homopolymer	—	50.0
Ethyl acrylate homopolymer	7.0	—
Ethyl acrylate/methyl methacrylate (80/20)	15.4	18.5
Ethyl acrylate/methyl methacrylate (70/30)	17.2	20.6
Ethyl acrylate/methyl methacrylate (25/75)	67.0	38.6
	Ethyl acrylate	*Butyl methacrylate*
Butyl methacrylate homopolymer	—	95.5
Ethyl acrylate/butyl methacrylate (50/50)	36.8	59.3
	Ethyl acrylate	*Styrene*
Styrene homopolymer	—	48.6
Ethyl acrylate/styrene (77/23)	19.1	41.6
Ethyl acrylate/styrene (7/93)	25.0	57.0
	2-Ethylhexyl acrylate	*Methyl methacrylate*
2-Ethylhexyl acrylate homopolymer	12.0	—
2-Ethylhexyl acrylate/methyl methacrylate (60/40)	19.0	39.0
2-Ethylhexyl acrylate/methyl methacrylate (40/60)	33.6	50.0
	Butyl acrylate	*Methyl methacrylate*
Butyl acrylate/methyl methacrylate (80/20)	11.4	21.8
Butyl acrylate/methyl methacrylate (20/80)	36.4	42.6
	Styrene	*Methyl methacrylate*
Styrene/methyl methacrylate (7/93)	96.0	63.0
	Butyl methacrylate	*Methyl methacrylate*
Butyl methacrylate/methyl methacrylate (60/40)	97.0	50.6

TABLE IV

Ratio of monomers in the polymer (A)		*Ratio of peak areas of respective monomers evolved (B)*	*Ratio A/B*
Ethyl acrylate/styrene			
(77/23)	3.35	0.43	7.8
(7/93)	0.075	0.01	7.5
Ethyl acrylate/methyl methacrylate			
(80/20)	4.0	0.92	4.35
(70/30	2.33	0.54	4.31
(25/75)	0.33	0.16	2.06[a]
Methyl methacrylate/butyl acrylate			
(85/15)	5.67	10.0	0.57
(75/25)	3.00	5.2	0.58
(65/35)	1.86	3.2	0.58

[a] In this case it is suspected that not all the ethyl acrylate was in polymeric form.

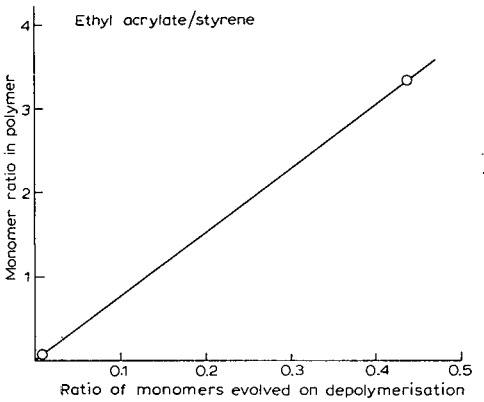


Fig. 7.

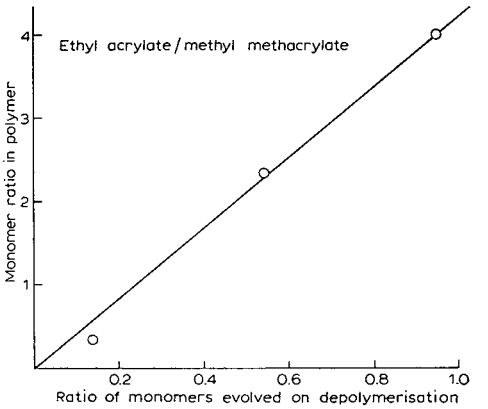


Fig. 8.

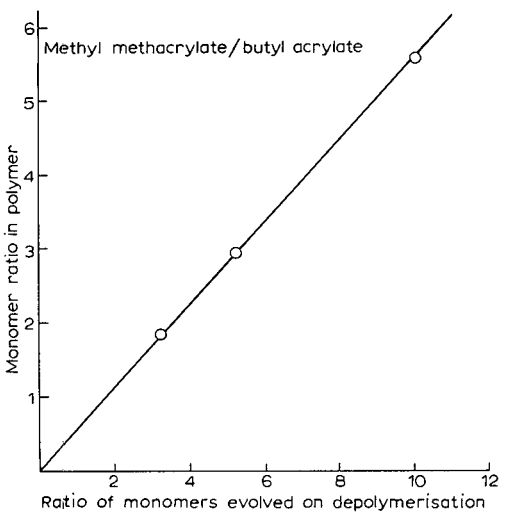


Fig. 9.

DISCUSSION

Mechanism of depolymerisation

On trying to explain the mechanism of depolymerisation some understanding of the thermal degradation process is necessary.

There are a number of possibilities that could govern the thermal degradation of polymers:

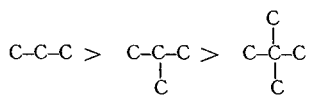
(1) The bonds in the chain may be broken at random, each bond being of equal strength and accessibility[4].

(2) There may be a number of weak links in the chain which are more easily ruptured than the normal bonds[5].

(3) The weak links may induce reactions which activate the weak points and split off a number of monomer units[6].

(4) The degradation may be caused by an activation of the chain ends followed by a rapid splitting off of the monomer units (propagation reaction)[7].

The thermal stability of a polymer and the breakdown products obtained on pyrolysis may be related in part to the relative strengths of the –C–C– bonds, thus:



In the case of polyethylene chains, experimental results show that the more hydrogen atoms in the chain are substituted and the larger these side groups, the greater the monomer yield on depolymerisation. According to MADORSKY[9] polyethylene has a monomer yield of 0.03 %, polypropylene 0.17 %, polyisobutylene 18.1 %, polystyrene 40.6 % and polymethylstyrene 100 %.

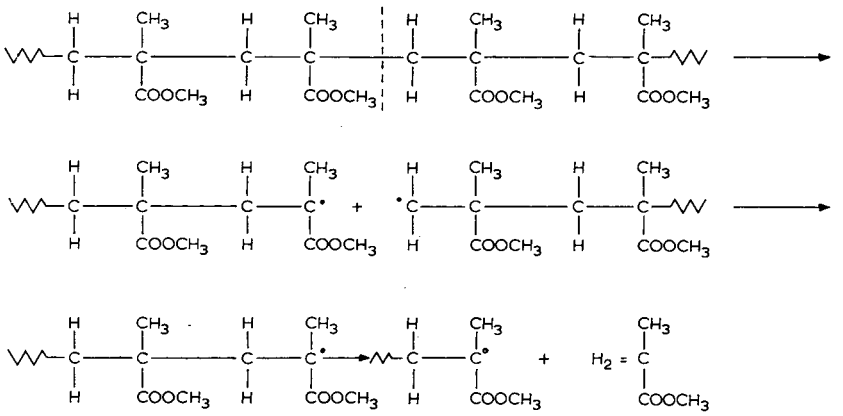
GRASSIE AND MELVILLE[10] investigating the thermal degradation of methyl methacrylate polymers of various molecular weights found that for a polymer of molecular weight of 44,300 (I) the molecular weight of the residue remained constant up to 65 % degradation.

In the case of polymers of molecular weight 94,000 (II) and 179,000 (III), the molecular weights of the residues remained constant up to 30 % and 20 % degradation respectively after which they dropped sharply to approximately 70–80 % of the original value at about 60 % degradation.

In the case of a polymer of molecular weight 725,000 the molecular weight of the residue dropped linearly to 40 % of the original value at 60 % degradation. HART[11] similarly found that a polymer of molecular weight 5,100,000 at 10 % degradation gave a residue with a molecular weight of one-third of its original value.

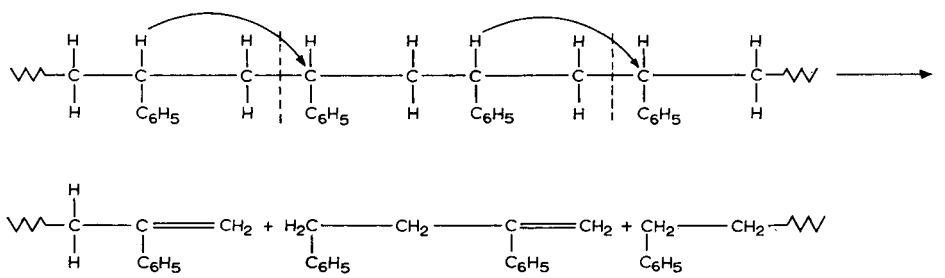
This would indicate that the higher the molecular weight of the polymer the greater the drop in the molecular weight of the residue on partial depolymerisation. According to GRASSIE AND MELVILLE[10] this degradation behaviour of high molecular weight polymers indicates a random scission followed by an unzipping process where monomers split off the chain ends one at a time. In the case of polymers, (I), (II) and (III) above, the assumption is that a steady state is reached in the first stage of depolymerisation because of the rapid disappearance of the short chain polymer. It would thus appear that there are two mechnisms at work and this might explain the double peaks obtained in some stepwise traces (Figs. 3–5).

The degradation of polymethyl methacrylate is thus believed to be mainly governed by random scission. These scissions are not accompanied by a hydrogen transfer because of the steric hindrance of $-CH_3$ and $-COOCH_3$ on every alternate carbon which is quaternary. The scission thus results in the formation of free radicals which once initiated unzip completely. In this way only monomers appear in the pyrolyzate.



In the case of polystyrene, STAUDINGER AND STEINHOFER[12] examined the degradation and found that in addition to monomer, dimers and higher units were also formed and postulated a mechanism of scission at weak links to explain the formation of such units.

Since the bonds between hydrogen and tertiary carbon are weaker than those between hydrogen and secondary carbon the formation of fragments larger than monomer takes place:



Similarly higher units may be formed. However, not all scissions result in unsaturated ends. In some cases the break results in free radicals which will unzip to yield monomers. Thus, styrene polymers, in addition to chain scission at weak links, also depolymerise by an unzipping process and consequently monomers, dimers and higher units are formed.

Support of the theory of weak links in the case of polystyrene is given by GRASSIE AND FARISH[15]. They examined the molecular weight of the residue on degrading a polymer of molecular weight 73,000 and found that after 20 % degradation the molecular weight dropped to just below 20 % of the original, the molecular weight falling very sharply in the initial stages of depolymerisation.

It is interesting to note that polystyrene both on stepwise and one shot pyrolysis gave similar results (49 % monomer yield, see Table II). This figure is in reasonable agreement with the theoretical value of 56 %[15].

In the case of acrylate polymers the degradation mechanism does not favour the

formation of free radicals as in methacrylates. STRAUS AND MADORSKY[13,14] studied the thermal degradation of polymethyl acrylate by mass spectrometry and found the monomer yield to be 0.7 % of the total volatiles among which were considerable amounts of carbon dioxide, methanol and long-chain fragments. Our results by pyrolysis/GLC of acrylates show the respective alcohol to predominate over the monomer but the yield of the latter depends on the depolymerisation conditions. The yield obtained by one shot was higher (6–12 %) than that obtained by stepwise depolymerisation (2.5–6 %).

From what is known of the degradation of homopolymers, it is anticipated that the degradation behaviour of copolymers would be dominated by that of the major constituent. The extent of these influences in the case of thermal degradation of styrene/methyl methacrylate copolymers has been studied by GRASSIE AND FARISH[15]. It was found that the number of weak links in the copolymer was proportional to the amount of styrene and that a sequence of at least ten styrene units was necessary in the copolymer molecules to produce dimers, trimers and tetramers. As a consequence a higher monomer yield of styrene was anticipated from copolymers with low styrene content.

Using the reactivity ratios based on the copolymerisation theory[16,17] they calculated the sequence of styrene units present in the copolymers used.

It was thus worked out that for a 1/4 styrene/methyl methacrylate copolymer 87 % of the polymer contained single units and 11.7 % double units. For a 10/1 styrene/methyl methacrylate copolymer only 1.0 % were single units and 71.9 % were above ten units.

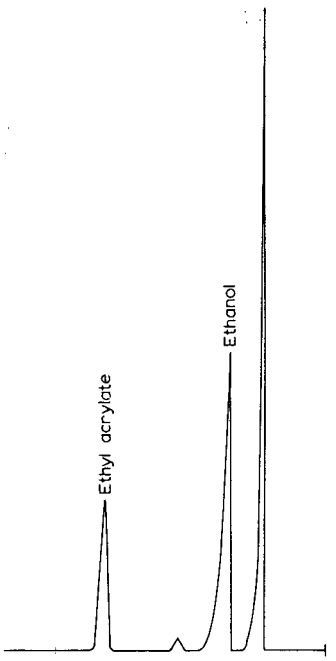
If it is assumed that all styrene sequences up to ten units yield only monomers and above ten give the characteristic breakdown of pure polystyrene, the monomer yield for 10/1 styrene/methyl methacrylate would be 60 % and for 1/4 styrene/methyl methacrylate nearly 100 %. In this work the yield obtained for a 7/93 styrene/methyl methacrylate copolymer was 96 %, which is in good agreement with the anticipated results.

It appears, therefore, that the major constituent in a copolymer plays a predominant part in the depolymerisation behaviour of copolymers and the results obtained in this work by pyrolysis/GLC (Table III) could be explained theoretically.

APPLICATIONS

A few selected examples are shown below:

Figs. 10–12 show the pyrograms of ethyl acrylate homopolymer and ethyl acrylate/methyl methacrylate copolymers 80/20 and 25/75, respectively. The traces were obtained by one shot pyrolysis at 450°, using a 4 m silicone oil column at 70°. Identical weights of polymer were depolymerised in each case (25 μg) and the instrument sensitivity was kept the same in all cases so as to permit a comparison of the amounts of various constituents evolved to be made. It may be seen that the ethyl acrylate monomer evolved by the 25/75 ethyl acrylate/methyl methacrylate copolymer is more than twice that evolved by the homopolymer although the amount of ethyl acrylate in the copolymer is one quarter that of the homopolymer. It may also be noticed that the amount of ethanol is considerably less in the 25/75 copolymer compared with the homopolymer.



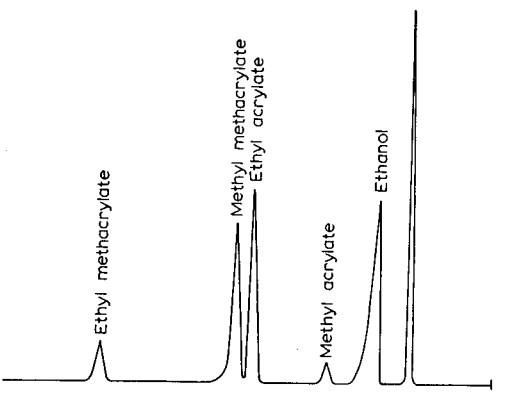
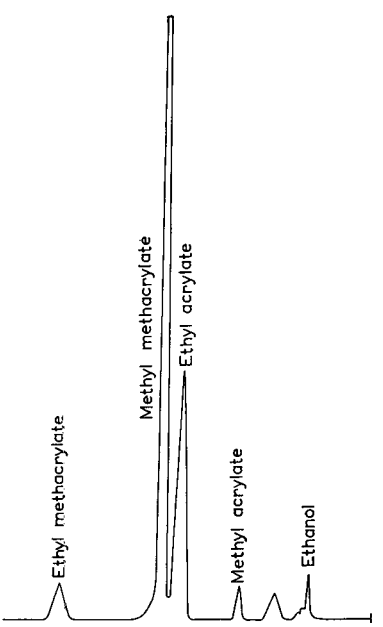


Fig. 10. One shot pyrolysis at 450°; 4 m silicone oil column at 70°. Polyethyl acrylate, 25 μg.

Fig. 11. One shot pyrolysis and 450°; 4 m silicone oil column at 70°. Ethyl acrylate/methyl methacrylate copolymer (80/20), 25 μg.



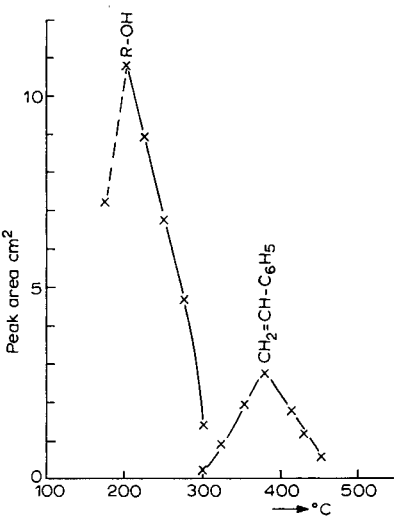


Fig. 12. One shot pyrolysis at 450°; 4 m silicone oil column at 70°. Ethyl acrylate/methyl methacrylate colpolymer (25/75), 25 μg.

Fig. 13. Stepwise depolymerisation of styrene/maleic monoalkyl ester.

Other noticeable points are the presence of methyl acrylate and ethyl methacrylate in the breakdown products of the copolymer. Whether this ester interchange takes place on depolymerisation or is effected in the initial copolymerisation is not yet known.

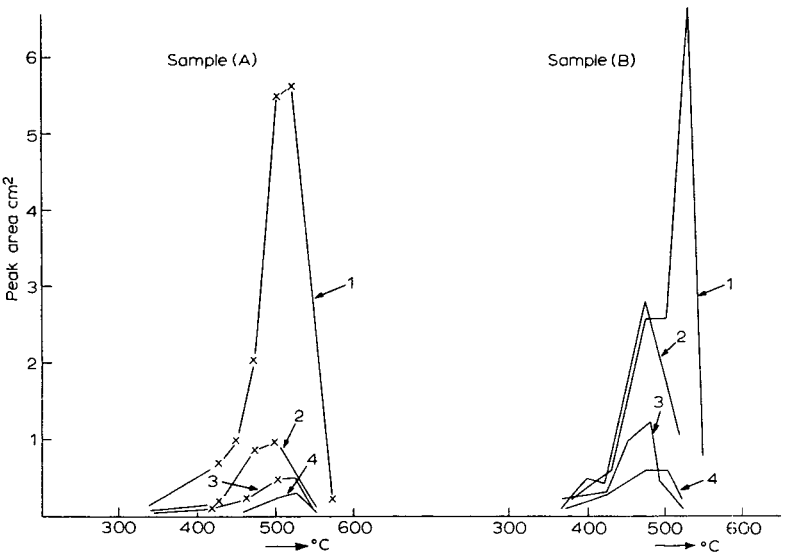
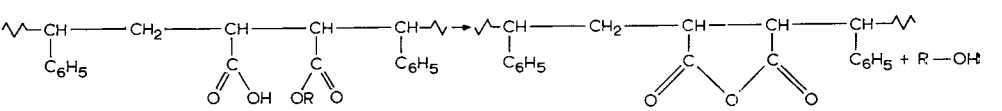


Fig. 14. Stepwise depolymerisation of unknown samples. 1 = Styrene; 2 = methyl methacrylate; 3 = butyl acrylate; 4 = butyl methacrylate.

Fig. 13 shows the stepwise depolymerisation of a copolymeric styrene/maleic acid monoalkyl ester. It indicates that the breakdown takes place in two stages:

First, elimination of the alcohol and the formation of anhydride. Secondly,



the breakdown of styrene/maleic anhydride copolymer.

Fig. 14 shows the stepwise depolymerisation of two unknown materials (A) and (B). Both samples showed on one shot pyrolysis styrene, methyl methacrylate, butyl acrylate and butyl methacrylate in various ratios. The stepwise depolymerisation, however, showed differences in styrene evolution which would indicate sample (A) to be a random quadri-copolymer, whereas sample (B) is either a mixture of polystyrene and quadri-copolymer or a block styrene copolymer with the quadri-copolymer.

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CHROM. 3891

HIGH-CAPACITY MULTI-CHANNEL COMPUTERIZED READ-OUT SYSTEM FOR MULTI-COLUMN CHROMATOGRAPHY

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SUMMARY

A computerized multi-channel read-out system for multi-column chromatography is described. It has a capacity of a hundred chromatograms per day when three channels are used and this can be expanded to four hundred chromatograms per day by the use of all twelve channels. The system is compared with other high-capacity systems for automated chromatography.

INTRODUCTION

We have previously described an automatic read-out system for multiple column chromatography connected via data-logger and paper tape output to a computer[1].

The development of a magazine-fed fraction collector for multi-column liquid chromatography[2] together with capillary column techniques for multi-column chromatography[3,4] has greatly expanded the potential capacity of multi-column systems for liquid chromatography. It has therefore been necessary to develop multi-channel automated read-out systems capable of handling the large numbers of chromatograms that can be produced by the new multi-column capillary systems.

GENERAL DESCRIPTION OF THE SYSTEM

The multi-channel read-out system is built around the recently described new type multi-collector[2]. The test tubes with the colored reaction mixture on which a transmittance reading is desired pass from the magazine to the left in the collector (Fig. 1) to the magazine on the right past a central latch that moves the tubes row by row past a set of suction tubes that dip in and out of the test tubes on signal from the central programmer. The reaction mixture is passed tube by tube through the colorimeters visible in the foreground and the transmittance is recorded on the multi-pen recorders to the right in the picture. These recorders are equipped with retransmitting slide-wires that send signals simultaneously to a data-logger and via the proper interphase systems directly on-line to a computer for data-handling.

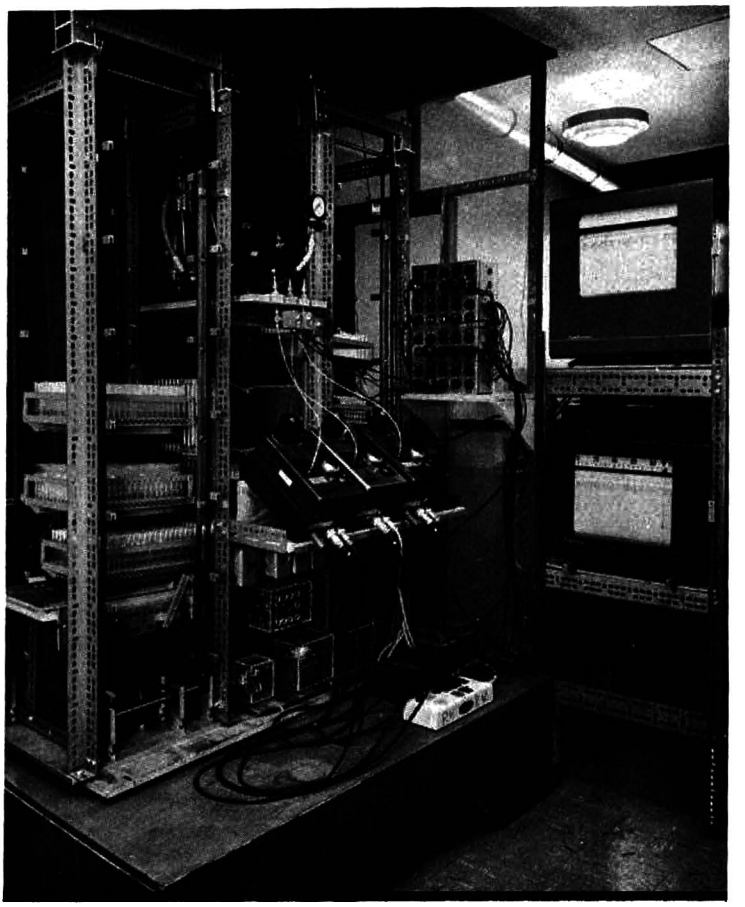

Fig. 1. The multi-channel read-out system set up as a 3-channel system. In the background the magazine-fed multi-column collector[2]. In the foreground the three colorimeters. The control panel (Fig. 3) is to the right above the colorimeters. A three-pen and a two-pen recorder are located on the rack at the extreme right. The suction "dipper" mechanism (Fig. 4) is behind the colorimeters and the controlled vacuum system (Fig. 6) is on the shelf above in the center of the multi-collector. The data-processing equipment (Fig. 7) is hidden behind the plastic sheet wall located in back of the control panel.

DETAILS OF CONSTRUCTION

Programming of events

The sequential steps in the automated read-out procedure are programmed through a punched tape programmer (Industrial Timer, Parsippany, N.J. No. 242) shown in Fig. 2. The program repeats sequentially twenty cycles, each consisting of the following steps: suction tube dips into test tube, valve opens to central vacuum to draw liquid through flow cell, valve closes to central vacuum, recorder drive is activated, switch to data-logger and computer system is closed, suction tube is lifted out of test tube, latch is activated to move a new row of test tube into position and so

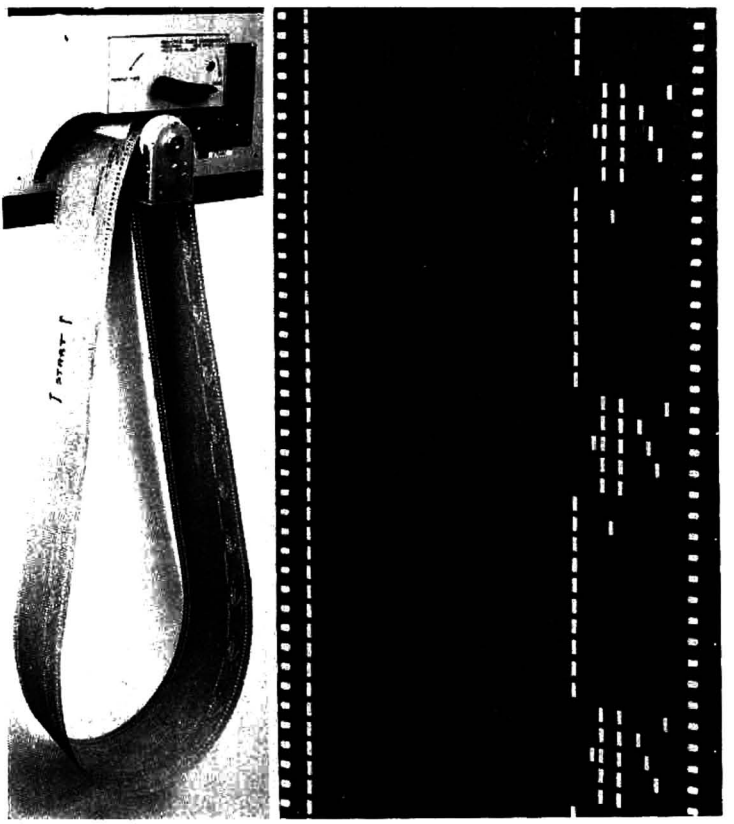

Fig. 2. The event programmer. To the left the timer with tape; to the right the programmed tape. A synchronous motor mechanism moves the tape from hole to hole each 1/2 sec and events are programmed by perforating the required number of holes in each channel. A contact closure is effected as electrical brushes pass over the perforations.

on. At the end of the twentieth cycle a longer delay is introduced after the latch has been activated to allow a new test tube carrier to move into position at the latch. The program is punched channel by channel by a special accessory to the tape programmer. The finished tape is shown to the right in Fig. 2.

The contacts made in the tape programmer when brushes travel over the holes punched in the tape can carry only a very moderate amount of current. We have therefore in all cases let this current activate a coil in heavy duty mercury relays mounted behind the plates in the central control panel shown in Fig. 3. We have also mounted on and off switches for each operation so that the impulse from the programmer can be passed on to or be withheld from the peripheral equipment as needed allowing single steps in the procedure to be isolated and tested separately.

Drawing the samples through the flow cells

We decided in the construction of the read-out machinery to use the colorimeters from the Technicon Autoanalyzer system primarily because these colorimeters have proven themselves over the years in day by day automated analyses in many labora-

tories the world over. The flow cells have been the tubular flow cells used in the Autoanalyzer N system. We have found that these flow cells can be used in our application without a debubbler if they are arranged at an angle of 45° to the horizontal as illustrated in Fig. 1. This is an important feature in the system since it has been found that air bubbles that inevitably occur now and then in the early part of the suction phase do not get trapped in the flow cell if it is arranged in this position.

We used in our earlier system[1] pumps to pump the liquid through the cells. This system has, however, over the years proven somewhat cumbersome needing a great deal of maintenance to function well. We have therefore switched to a simplified system in which a central vacuum is used. The arrangement is illustrated in Fig. 6. Vacuum from house vacuum or a small vacuum pump is adjusted through a Cartesian vacuum regulator (Manostat No. 6A, Greiner Scientific, New York, N.Y.) and the use of a bleed valve to the proper experimentally determined setting (approximately 15 in. mercury pressure). The regulated vacuum is connected to a large suction flask

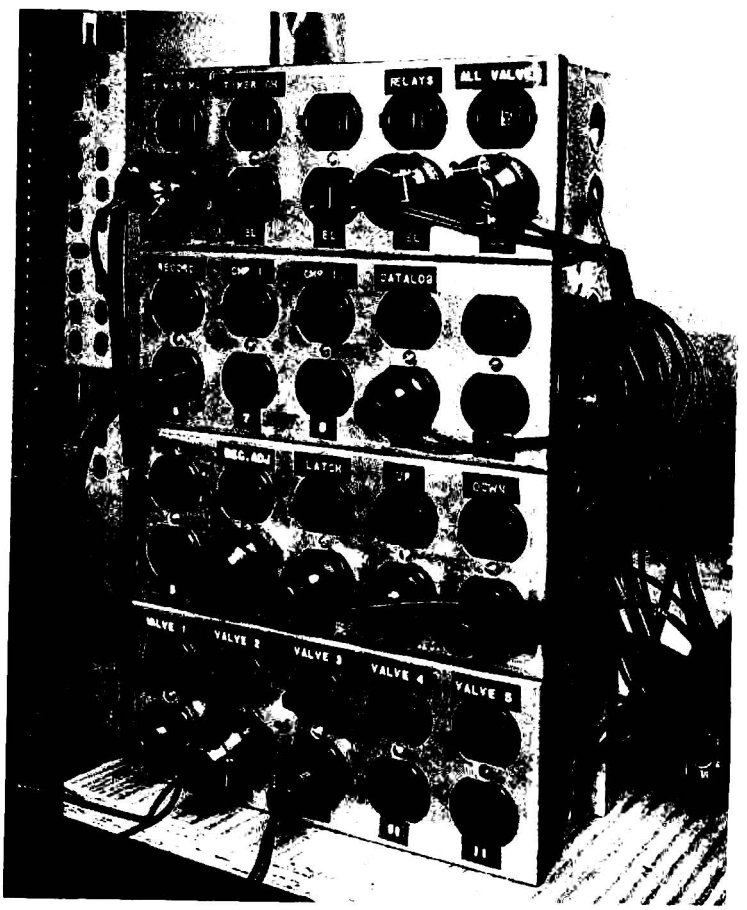

Fig. 3. The control panel. Wires from the event programmer are led to the coils in mercury relays located behind the switches. The loads are connected via switches to the load contacts on the mercury relays. All operations can be controlled from the switches on this panel.

from which it is distributed to individual flow cells through needle valves that regulate the flow and through on/off solenoid valves that open and close at command from the event programmer.

Teflon tubing and stainless steel connectors are used throughout in the suction line. Heavier (1/8 in. diameter) teflon tubing is used on the vacuum side of the flow cell. Thin teflon tubing (AWG No. 22) leads from the flow cell to the suction tube that dips into the liquid in the test tubes. The teflon tubing is carried through the inside of this piece of protective stainless steel tubing to the tip (Fig. 4).

The "dipper" mechanism

The suction tubes and the method of dipping into the test tubes are illustrated in Figs. 4 and 5. Fig. 5 shows the air cylinders used to push the teflon tube carrying stainless steel tubes into the test tubes and back up. They are double acting air cylinders (Clippard Inc., Cincinnati, Ohio No. 3BDS-6) that work on the principle that compressed air applied to the top side of the cylinder will drive a stainless steel rod downwards 6 in. Air applied to the bottom port of the cylinder will send the rod back up. A piece of stainless steel tubing is attached to this rod and the teflon tubing coming from the flow cell is passed through a hole in a short adapter connecting rod and stainless steel tubing and pushed through the inside of the stainless steel tubing

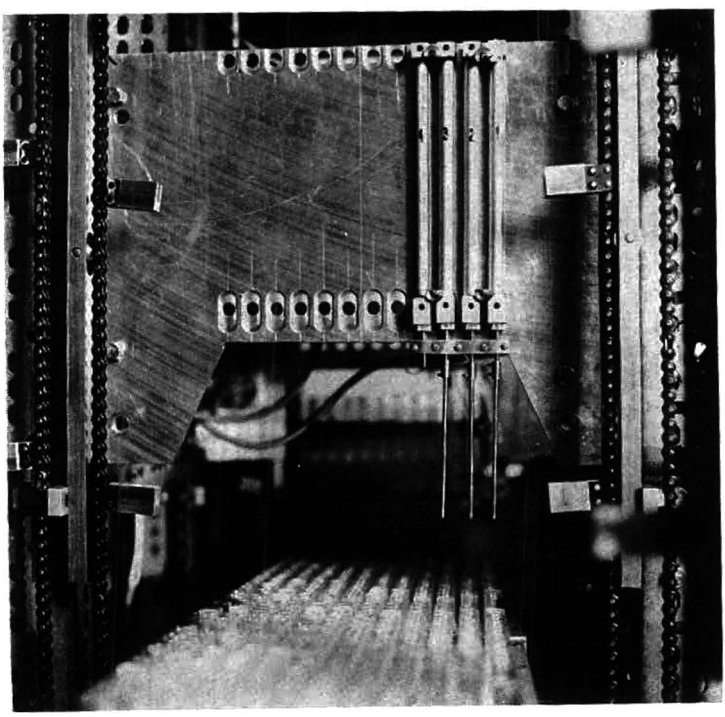

Fig. 4. The "dipper" mechanism. When compressed air is introduced at the top of the air cylinders (Fig. 5) the stainless steel tubes attached to the cylinders carry thin teflon tubing into the test tubes. When compressed air is introduced at the bottom of the cylinders the tubing moves out of the test tubes.

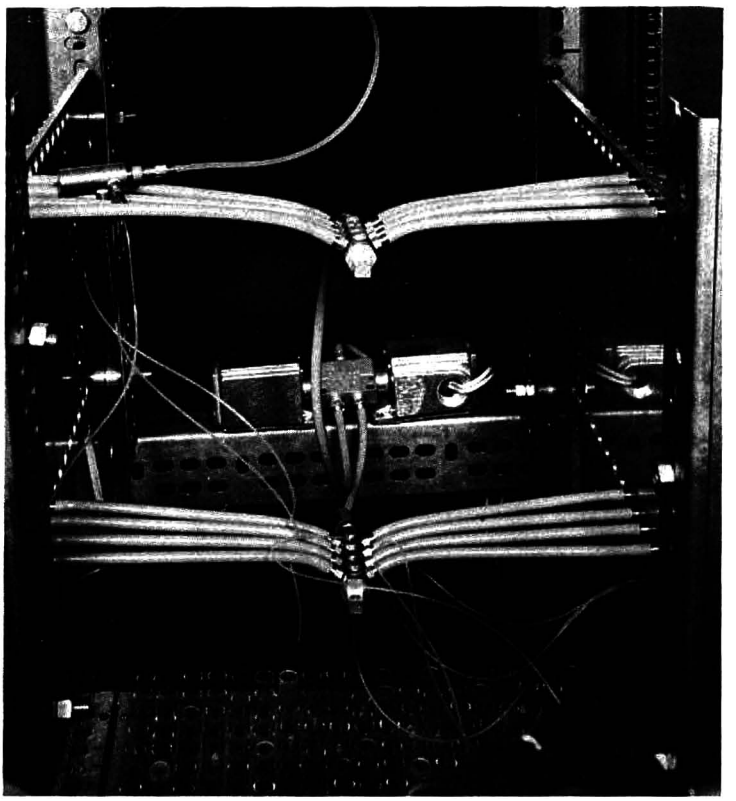

Fig. 5. The compressed air system. A solenoid valve in the background directs the air either to the top of the air cylinders (for dipping down into the liquid) or to the bottom of the cylinders (for retraction from the test tubes).

to the tip of this tubing. Through the valve and distributing system shown in Fig. 5 compressed air is sent first through the top port of the air cylinder by activating the right side of the solenoid valve (Clippard No. AVSC-115) shown in the background of Fig. 5. It is later returned to its "up" position by activating the left side of the solenoid valve opening the lower set of plastic distributing tubes to the compressed air.

The system as shown in the pictures has two sets of "dipper" mechanisms. One is used for bubbling carbon dioxide through the reagent mixture in the special reaction for 17-ketosteroids described earlier[5]; the second "dipper" mechanism is the mechanism used for sucking sample into the flow cells.

DATA-PROCESSING

The high capacity of the multi-channel automatic colorimeter unit cannot be fully utilized unless the calculation of the data is handled by computer. We have estimated that it will take five full-time clerks using ordinary electric calculators to calculate the 8-hour output from the machinery. Clearly much of the advantage of the high-capacity system would be lost without high-speed data handling.

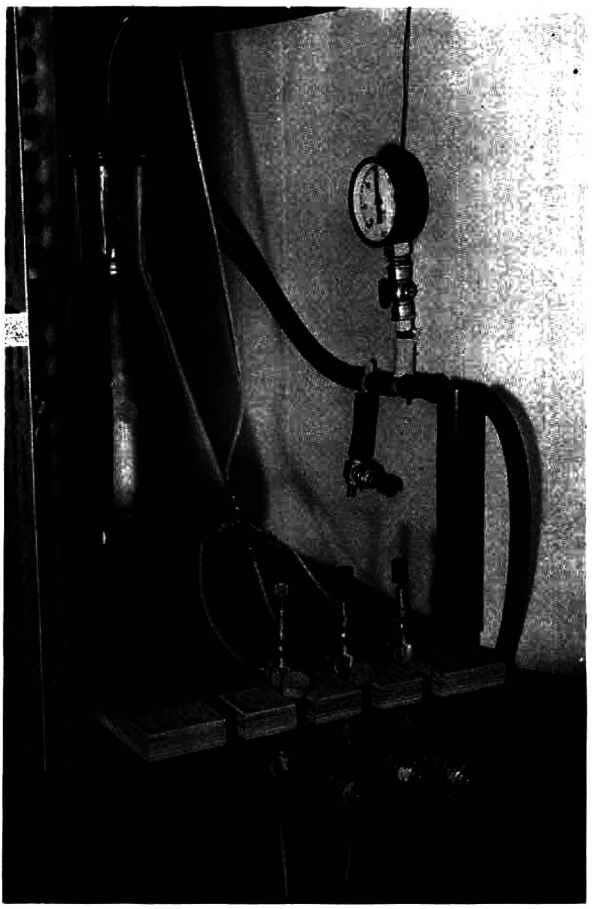

Fig. 6. The regulated vacuum system. The vacuum regulator (the black cylinder to the right) maintains in conjunction with the bleed valve located under the vacuum gauge the proper vacuum. A suction flask collects the reagent. Suction is applied to the flow cells in the colorimeters through the needle valves and solenoid valves shown at the bottom of the illustration.

We use for the data processing simultaneously two computer systems: a data-logger and a direct on-line system.

The data-logger (Fig. 7, top)

This is a commercial unit (Technilogger, Technicon Corporation, Chauncey, New York) that can receive data from up to eight colorimeters simultaneously and transform them to paper punch output at the same time labelling the input sequentially.

The on-line system (Fig. 7, bottom)

The signals from the retransmitting slide-wires in the multi-pen recorders are besides being passed to the data-logger also sent via a proper interphasing unit (Model AD-10, Technical Electronics, Bayside, New York) to an IBM model 1827 analog/

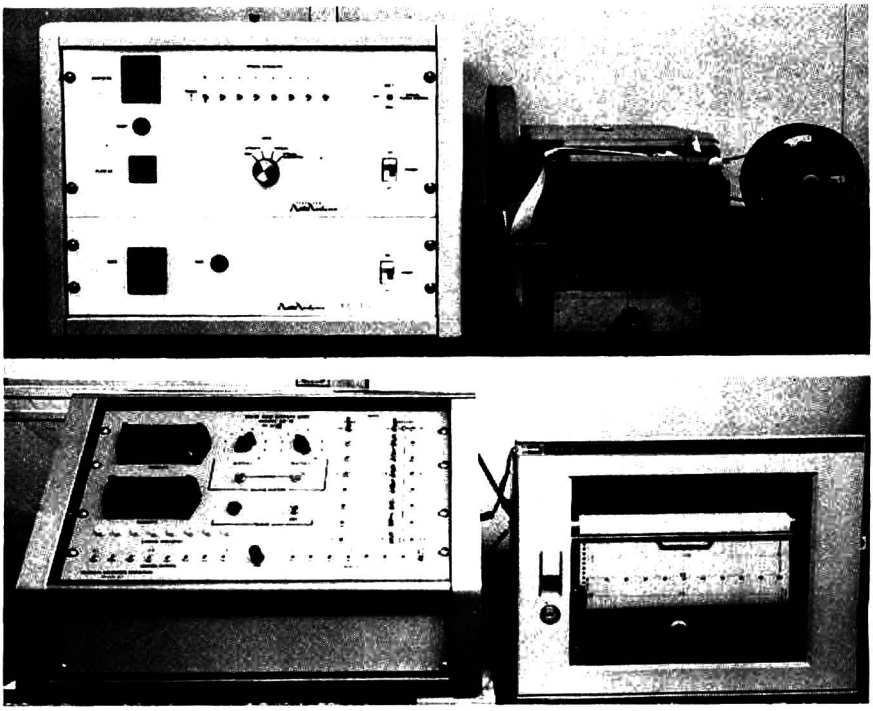

Fig. 7. The data handling systems. At the top the data-logger with its paper punch. At the bottom the on-line interphaser with a recorder for feedback of calculated data to the laboratory.

digital interphase for digitizing and from there to an IBM 360/30 computer for calculation. Calculated data can be transmitted back immediately to the laboratory and displayed on recorders in the laboratory.

Programming

The programs for the calculation of the chromatograms are currently under development and only the first relatively simple steps have been programmed. Peak values are summed and areas compared with similar areas for a set of simultaneously run standards to give amount of unknown present. The results are available as printed outputs and there are also several graphic outputs including plots of optical density values and micrograms compound per fraction cut sequentially registered to give a visual chromatogram to supplement the printed output.

The advantage of two independent computer systems

It may appear redundant to employ simultaneously two different computer systems. Experience has shown, however, that it is almost a necessity in high-capacity systems not to have to depend on a single system. We have not infrequently had computer breakdown lasting for a full working day. The data-logger paper tape has then been available for calculation once the computer was repaired and back in operation. Many man-hours of calculation time are saved this way. Running a double system also makes it possible to check one system against the other. Obviously

results from data-logger and on-line system must agree when both systems are functioning properly.

PERFORMANCE

We have in the performance testing of the apparatus concentrated upon the following aspects: reproducibility, carry-over, capacity and practicality of the system.

Reproducibility

The reproducibility of the combined multi-column system and the read-out machinery has been experimentally evaluated by chromatographing aliquots of mixtures of 17-ketosteroid standards using our routine system for multi-column chromatography of 17-ketosteroids[1,6] adapted to capillary columns. Approximately 50 μg amounts of each of the seven 17-ketosteroids dehydroepiandrosterone (DHEA), androsterone (A), etiocholanolone (E), 11-keto-androsterone (OA), 11-keto-etiocholanolone (OE), 11-hydroxy-androsterone (OHA) and 11-hydroxyetiocholanolone (OHE) corresponding to the main urinary 17-ketosteroids were chromatographed on twelve 6-foot capillary columns using capillary teflon columns[3] and gradient elution chromatography[6] in a simultaneous multi-column run.

We have for comparison on a commercial automated gas chromatograph (Barber-Colman) run a similar set of standards twelve times sequentially to compare the relative reproducibility of an automated liquid column system with that of the automated gas chromatograph. The conditions for the gas chromatographic assay were: column substrate, neopentyl glycol succinate 2% on Gas-Chrom P, mesh 100/120 (from Applied Science Lab., State College, Pa.) coated using the technique described by HORNING *et al.*[7]; hydrogen flow, 40 ml/min; air flow, 350 ml/min; column temperature, 220°; injector temperature, 250°; detector temperature, 250°; column length, 6 ft., I.D. 3 mm. Pregnane-3,20-dione was used as an internal standard. The steroids in the gas chromatographic assay were run as trimethylsilyl ethers prepared as suggested by KIRSCHNER AND LIPSETT[8]. The gas chromatograms were calculated in three different ways: by peak height multiplied by the peak width at half height, by the use of peak height alone and by multiplying peak height by retention time for a given compound, in all cases comparing with the internal standard.

The last of these methods gave the highest accuracy in the calculations and has been used for the comparison with the liquid system.

It can be seen from Table I that the reproducibility is clearly better for the automated liquid chromatography. The automated gas chromatography although considerably less reproducible must still be considered quite adequate for most biochemical work.

Carry-over

Since teflon tubing dips down into the reaction mixture in the test tubes and moves from tube to tube it was considered necessary to experimentally evaluate the possible carry-over from tube to tube. The carry-over consists of two factors. One is the mixing with the previous sample as new sample is drawn into the flow cell. The other is caused by sample sticking to the outside of the teflon tubing. The first factor can obviously be eliminated by drawing sample long enough through the flow cell until

TABLE I

COMPARISON OF THE REPRODUCIBILITY OF AUTOMATED LIQUID CHROMATOGRAPHY AND AUTOMATED GAS CHROMATOGRAPHY

In both cases twelve chromatograms of seven 17-ketosteroids were run and means and coefficient of variations (standard deviation in % of the mean) calculated.

	Coefficient of variation (%)							
	DHEA[a]	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*	
Liquid chromatography	2.5	2.0	1.2	3.0	2.6	1.2	1.3	av. 2.0
Gas chromatography	2.6	3.1	3.1	2.9	3.1	2.5	7.2	av. 3.5

[a] For abbreviations for individual steroids see text.

all traces of old sample have been washed out. The other factor was found to be negligible because of the surface characteristics of the teflon. The relationship between volume sucked through the cuvette and carry-over was found to be as follows: at a suction volume of 0.8 ml a carry-over of 8.7 % was found, at a suction volume of 1.2 ml the carry-over was 0.8 %, and at a suction volume of 1.7 ml no measurable carry-over could be determined indicating that it was at least below 0.2 %. This then would be maximum carry-over produced by the teflon tubing dipping into the solutions in the test tubes.

Capacity

The aim in the development of the new system was to construct a high-capacity system. This has been obtained through the use of multiple channels. Some increase in capacity has also been gained through the use of vacuum suction instead of the pumps previously used to get the samples to the flow cells.

The current 3-channel system has a capacity of approximately 100 17-ketosteroid chromatograms per working day when 60 test tubes per chromatogram are read and estimating the seven main urinary 17-ketosteroids.

It is expandable to a 12-channel system by installing a total of twelve colorimeters with the necessary extra multi-pen recorders. The potential capacity of the system is therefore 400 chromatograms per working day when applied to a biological 17-ketosteroid mixture.

Practicality

The apparatus has been in routine use over the last twelve months in our laboratory. It has after initial "debugging" been found quite reliable with little maintenance needed. No specially trained personnel is needed for the operation of the instrumentation. Female laboratory technicians without special background in handling of instruments have been running the routine chromatograms on the machinery through these months.

DISCUSSION

Recent years have seen chromatography dominated by an explosive growth of gas chromatographic and thin-layer methods. Other chromatographic methods have been pushed somewhat into the background during this development.

It is our contention that the potential of column chromatography with proper upgrading and automation of procedures and a switch towards high resolution, relatively fast, multi-column capillary column techniques is fully as great as that of the newer techniques although no one technique can be expected to be the method of choice for all applications. Liquid–liquid and liquid–solid column chromatography will, however, we believe still be found in many cases to be the preferable chromatographic method.

The multiple capillary column techniques have been developed to overcome two of the very real shortcomings of column chromatography in practical work: low capacity and slow speed. With the new automatic read-out apparatus described here a factual capacity of 100 chromatograms and a potential capacity of 400 chromatograms per day has been reached. We have currently in our laboratory the capacity to do 100 capillary column separations in a day using our special multi-column fraction collectors. We can do this by starting one set of 50 columns in the morning and setting up another set in the afternoon. The investment in chromatographic pumps to achieve this capacity has, however, been high and for that reason new greatly simplified systems that eliminate the chromatographic pumps are currently under development. We believe that it will be possible in some routine chromatographic separations to expand from a hundred to several hundred capillary column chromatograms per day with these new systems without excessive demands on space and facilities.

To our knowledge the only other chromatographic system that can produce chromatograms and calculated values for 100 chromatograms per day or more is the "Cassandra" system for automated paper chromatography described by BUSH[9]. It is, we believe, of interest to compare the two systems on a number of points of importance for the performance of the total systems to give the chromatographer in search of high capacity automated systems a guide to the system best suited for a given application.

Comparison of the multi-column system with Bush's "Cassandra" system for high capacity chromatography

Resolution and speed. Techniques for liquid chromatography have traditionally been by far the slowest of all chromatographic techniques. New developments over the last decade have, however, changed the situation radically.

The first to systematically explore experimentally and theoretically the possibilities for fast liquid chromatography was HAMILTON[10,11], who showed that the separation of amino acids on ion-exchange columns could be significantly accelerated by the use of high pressure and together with his collaborators[12] studied a number of the variables affecting the chromatographic resolution. The theoretical potential of capillary columns in liquid chromatography was pointed out by GIDDINGS[13] who showed that theoretically the separating ability is higher in liquid chromatography than in gas chromatography since the theoretical limit to the number of plates is roughly 1000 times larger in liquid than in gas chromatography. Recently SNYDER[14,15] has evaluated the problem of maximum resolution per unit time in liquid–solid adsorption chromatography experimentally and theoretically and evolved equations and charts for maximum bed efficiencies in this type of chromatography as a function of column pressure, column length, separation time and particle size. The highest theoretically possible bed efficiencies cannot currently be realized in liquid–solid

chromatography because of technical limitations in pressures that can practically be obtained but as pointed out by SNYDER[14,15] a maximum number of 60,000 theoretical plates can conveniently be obtained today without exceeding known technology. Long runs (72 h) are, however, necessary for this. Resolutions equal to that obtained with packed columns in gas chromatography and far better than resolutions obtainable in thin-layer and paper chromatography can, however, be realized in high pressure, fast speed chromatography. SNYDER[15] in his experimental work reached bed efficiencies in 40 min of more than 2000 theoretical plates in the separation of hydrocarbons and he reports plate numbers of over 4000 in 2 1/2-hour runs working with a synthetic mixture of hydrocarbons separated on 32 ft. long columns at 10 atm. pressure. We have (unpublished) with the capillary technique described earlier[3,4] in routine work realized theoretical plate numbers of about 4000 in 4-hour runs separating corticosteroids on 6 ft. long columns at 30 atm. of pressure. Fast high-pressure capillary column chromatography of nucleotides was performed by HORVATH *et al.*[16] on 6 ft. columns and at 75 atm. pressure using pellicular column materials with separation of complex mixtures possible in from 75 to 90 min. These authors draw the overall conclusion that this liquid chromatographic technique is comparable in speed, resolution and quantitative range to gas chromatography.

It is clear from these examples of high-pressure long-column chromatography that practical techniques now exist that make fast column runs possible and with much better resolution than is possible in thin-layer and paper chromatography as pointed out by SNYDER[14,15]. Liquid chromatography is getting close to packed column gas chromatography in speed for similar resolution as demonstrated by HORVATH *et al.*[16]. The very interesting work by PIEL[17] with high-pressure small-particle liquid chromatography in which high plate numbers were realized in very fast runs, would indicate that we are only at the threshold of further developments in this area and that with future improvements in technique liquid chromatography may overtake packed column gas chromatography in speed for similar resolution.

The better resolution in the multi-column capillary column system will, we feel, for many applications be found to be its most important advantage when compared with BUSH's automated paper chromatography. A calculation of theoretical plate numbers for the paper chromatographic estimation of the corticosteroids cortisol, tetrahydrocortisone and tetrahydrocortisol in BUSH's system was performed by TAIT AND TAIT[18]. Theoretical plates under optimal conditions were found to be 750 for levels below 40 μg and 390 for levels between 40 and 100 μg. We found running 50 μg samples of the same corticosteroids using the multi-column system with capillary columns[4] and using gradient elution chromatography that we in 4-hour runs could reach theoretical plate numbers of 3800–4200 for these same substances. The very much higher resolution in the multi-column system is clear. That the higher resolution is an important feature in practical chromatographic work is shown by the fact that urinary 17-ketosteroids can be separated quite well by a single column run on alumina in the multi-column system, but it takes two separate paper chromatograms and a Girard separation to effect the same separation in BUSH's system[19].

Choice of detectors. Another important advantage multi-column systems have when compared with automated paper chromatography is the wide choice of detectors available. If stream-splitting is used in the column chromatography a number of detectors can be applied to portions of the same fraction, for example, colorimetry,

fluorescence and liquid scintillation counting. The flame ionization detector used in gas chromatography has become available as a detector in liquid chromatography[20] as has a very sensitive U.V. detector[21]. It is obviously also possible to use the BUSH[9] system as a detector in column chromatography letting the effluent from a chromatographic column form spots on paper that then can be treated and quantitated in the "Cassandra" apparatus. In situations where the precision of the paper chromatographic system is adequate this could become a powerful combination utilizing the potentially higher resolution of the liquid column systems and the convenience of the paper system. The processing speed in the Cassandra apparatus great as it is would, however, have to be further accelerated before this would become a practical proposition for high capacity work.

The Cassandra system is in comparison more limited in choice of detectors. Besides some reagents are incompatible with the system and it is sensitive to changes in temperature and humidity.

Load. It has long been recognized that column chromatography has a decisive advantage here compared with other types of chromatography and this is, for example, shown in experimental studies like the one by TAIT AND TAIT[18] comparing paper and column chromatography of steroids. Much higher loads were possible on the columns without resolution being affected and impurities had less of an effect on the chromatography.

Reproducibility. Although great strides have been made in improving the precision of the method for automated paper chromatography values for coefficient of variation for urinary steroids estimated with the Cassandra system would seem clearly higher than similar values obtained in the multi-column system. BUSH[19] gives values of 4.2–8.2 % for the coefficient of variation in batch operation of biological samples for some common urinary steroids against the 3–5 % we have obtained routinely in similar experiments with the multi-column system. It must, however, be admitted that the increase in precision obtained by BUSH with the many refinements in technique over the years makes his automated paper chromatography system adequate in reproducibility for most practical applications.

Analytical capacity and practicality. Although as indicated we should shortly in our laboratory have the capacity to run several hundred capillary column chromatograms in a day it must be conceded that this with current techniques will require considerably more space than a similar-sized operation involving paper chromatography since a medium-sized room would have to be dedicated to column chromatography to make the higher capacity possible. A minimum of four large vacuum ovens will be necessary for evaporation of solvents and relatively large amounts of solvents will be needed per day, for example, with current techniques an estimated 24 l of benzene per 100 capillary column chromatograms of urinary 17-ketosteroids. Clearly paper chromatography is less demanding in space and solvents at this time although high-capacity work with this technique is rather space consuming also. It is also possible to recover much of the solvents used by trapping the solvents on evaporation.

It should be emphasized that the further development of microparticulate techniques for column work[17] may make a drastic miniaturizing of procedures possible when the aim is to obtain as practically as possible as high capacities as possible in liquid chromatography with maintenance of high resolution. Space and solvent demands may with such techniques drop to a fraction of what is currently needed.

Apart from requiring more space and more of an expenditure in solvents and reagents we believe that the multi-column technique otherwise in convenience and speed in preparing the chromatograms is fully as practical and fast as the paper chromatographic technique. The transfer step is, we believe, easier to automate in a column system than in paper chromatographic techniques. Even with manual syringe transfer techniques, however, the 20–25 chromatograms per hour given as the effective production rate of a good technician in the paper chromatographic system[19] can without strain be duplicated by a technician working with the multi-column system if machine filling[4] is used for the columns. We believe that the time currently spent taking tubes in and out of the vacuum ovens and adding reagent automatically with an automatic syringe pipette roughly equals the time spent running the BUSH system's machinery for the automatic preparation of paper chromatograms and that running the scanner is about equal in complexity to running our read-out machinery.

Choice of system. The chromatographer deciding between the multi-column system and the Cassandra system for high capacity chromatography would as major considerations for a given application have to weigh the better resolution, higher precision and wider choice of detectors in the multi-column system against the lower cost per analysis and smaller space requirements of the automated paper chromatography system. Obviously a great number of other factors would also enter into a decision about what system to choose. Skill of personnel, familiarity with particular procedures, availability of system components, system complexity, maintenance problems, computer access and economic factors are but some of the factors to consider when selecting a system. There is no obvious best choice for all applications.

The multi-column system compared with other chromatographic systems

Although no other high-capacity chromatographic system than the "Cassandra" system has been built it is obviously possible by investing enough in the necessary equipment to get high capacity in other chromatographic systems.

The commercially available automated gas chromatograph (Barber-Colman) can theoretically give a capacity of about twenty-two 1-hour chromatograms within a 24-hour period although we have found it not quite reliable enough to leave running unattended overnight. By combining four such chromatographs one can reach a capacity of close to 100 chromatograms per day.

The relative advantages of a 100 chromatogram multi-column system compared with such a system would be: no need for derivative formation, wider choice of detectors with higher specificity possible through the use, for example, of colorimetric and fluorescence reactions, higher load, cheaper apparatus and higher reproducibility with about equal resolution in the two systems as long as ordinary packed columns are used in the gas chromatograph. The advantages of the gas chromatograph would be: faster analysis time for the first of a batch of analyses, higher sensitivity in some applications, lower cost per analysis, convenience of being able to use a commercially available unit.

Systems could be set up using commercially available thin-layer scanners. Compared with such systems the multi-column system would have the same relative advantages and disadvantages as in the comparison with the automated paper chromatographic techniques.

Further developments

We have as mentioned newer simplified systems for multi-column chromatography under development in an attempt to economically reach the high capacity necessary in the chromatographic step to keep up with the expanded read-out capability. Also under development are simplified colorimetric channels using an inexpensive colorimeter/spectrophotometer instead of the colorimeter used in the current system. This will be cheaper per channel and will eliminate the expense of filters. In all this work as in the current apparatus commercially available apparatus is modified for inclusion in the combined system.

We hope later on to construct a special multicuvette spectrophotometer module that would use a 25 cuvette rotating cuvette arrangement and a single spectrophotometer and recorder to give 25-channel read-out capacity. This would be much cheaper per channel than the system described here since the cost of such a unit would be basically that of a spectrophotometer and a recorder plus the special construction cost involved. We hope also to construct a read-out system for high-specificity colorimetry which will include fast scanning of a pertinent part of the absorption curve, data recording on magnetic tape and computer analysis of the absorption curve.

ACKNOWLEDGEMENTS

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CHROM. 3876

USE OF A LIQUID CHROMATOGRAPH IN LIPID CLASS SEPARATION

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SUMMARY

The advantages of monitoring lipid separation on a silicic acid column with a liquid chromatograph have been demonstrated and discussed.

The recovery yields of known synthetic lipid mixtures have been shown to be good and the machine has been used for monitoring the separation of lipid classes from normal human blood plasma and serum samples.

It has been shown that in general it is not advantageous to separate neutral lipids from phospholipids prior to complete fractionation, and that the analysis of plasma lipids is more meaningful than that of serum lipids.

INTRODUCTION

The use of silicic acid in the separation of lipids into their respective classes has been a widely used technique for many years[1–4]. In the past this has involved testing each fraction for cholesterol[5,6], fatty acids[7–9], phospholipids[10,11] and in some cases for glycerol residues[12] and ester linkages[13]. In order to ensure complete elution of each fraction it was often necessary to pass excess solvent through the column.

The results reported in this paper were obtained by coupling a silicic acid column to a Pye System 11 Liquid Chromatograph. The purpose of this unit is to detect any organic material eluted from the column and to record its presence on a chart recorder. Fig. 1 gives a diagrammatic representation of the chromatograph. A moving steel wire passes from the feed spool to the cleaner oven where any dirt or grease is removed from the wire. In the coating block it passes through the column eluent and becomes coated with it. The solvent is removed in the evaporator oven and the solute in the pyrolyser oven. From the pyrolyser oven the solute passes, in a carrier stream of inert gas into a hydrogen flame ionization detector from where a signal is passed through an ionization amplifier to a moving chart recorder.

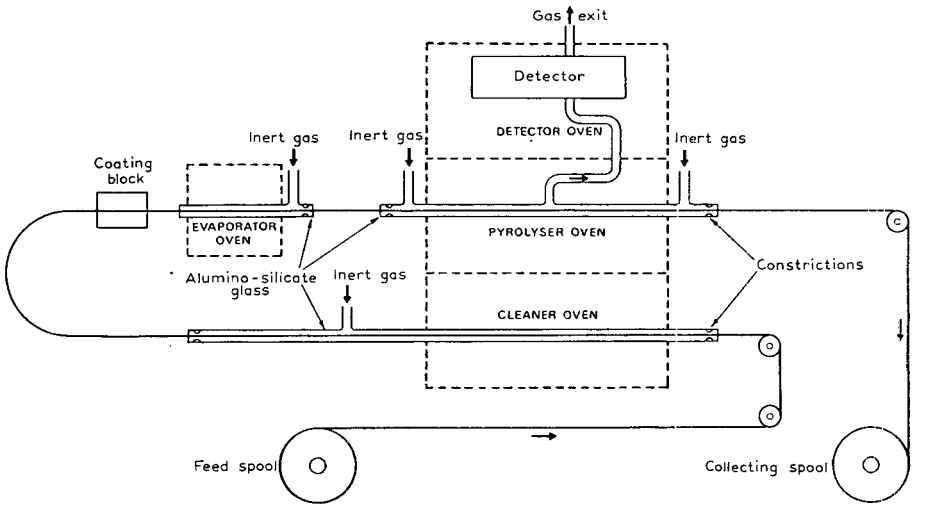

Fig. 1. Schematic diagram of liquid chromatograph.

EXPERIMENTAL

Materials

Silicic acid (Mallinckrodt) was washed several times with water followed by methanol and ether and allowed to dry at room temperature before being activated at 120° for 24 h. The silicic acid, now ready for use, was stored in a dry bottle with a tightly fitting cap. Columns were prepared immediately before use by pouring a suspension of silicic acid (1 g) in petroleum ether (b.p. 40–60°) into a 5 mm diameter column. The column was packed under pressure, and eluted with increasing concentrations of ether in petroleum ether and finally with methanol.

Analar grade solvents were used throughout. Biochemical-grade palmitic acid and cholesterol (B.D.H.) were used, and samples of cholesterol palmitate, tripalmitin, diolein, monopalmitin and L-α-lecithin (commercial grade) were obtained from Sigma Chemical Company.

Lipid extraction

Blood samples were processed immediately after withdrawal from the patient. Plasma samples had a 3.8 % citrate solution added to them (1 ml/10 ml blood) while serum samples were allowed to clot by standing at 37° for 1 h in the absence of citrate solution. Red cells were removed by centrifugation (3,500 r.p.m., 15 min).

Lipids were extracted from plasma or serum by a modification of the method of FOLCH *et al.*[14]. To ensure complete extraction the MeOH–CCl_3H mixture was refluxed for 5 min.

After extraction the lipid solution was evaporated to dryness, taken up in petroleum ether (*ca.* 1 ml) and stored at 4° for not more than two days.

Lipid assays

Cholesteryl derivatives. Cholesterol and cholesteryl esters give a green coloration with acetic anhydride in the presence of concentrated sulphuric acid[5]. Acetic anhydride (2 ml) was added to samples of cholesteryl compounds (0.1–2.0 μmole) in chloroform (5 ml) followed by concentrated sulphuric acid (0.1 ml). The colour was allowed to develop in the dark and measured at 630 mμ. The colour formed was found to be unstable and gave an optimum absorbance after 12–15 min. The most convenient method of obtaining reproducible results was by making repeated absorbance measurements until a consistent decrease was observed. The maximum absorbence value was recorded in each case and the method calibrated against a standard cholesterol solution.

Fatty acids. Glycerides (0.05–1.00 μmole) were hydrolysed as follows. The solutions were evaporated to dryness and then taken up in an ethanol–water (94:6, v/v) mixture (5 ml) containing two pellets of KOH (*ca.* 150 mg) and refluxed for 1 h.

After cooling and neutralising (HCl) the free fatty acid was extracted from the aqueous layer with hexane[7], and determined colorimetrically by the method of DUNCOMBE[8].

Phospholipids. Phospholipids (0.5–5.0 μmole) were completely hydrolysed and the inorganic phosphate liberated was determined by the method of FISKE AND SUBBAROW[15]. Hydrolysis was carried out by evaporating the sample to dryness in a Pyrex tube, adding 10 *N* H_2SO_4 (0.5 ml) and heating strongly for 5–10 min. It was found advisable to cover the open end of the tube with an inverted funnel connected to a water pump, as considerable fuming occurred. Any charred material was removed by adding a few drops of 20 vol. H_2O_2 and reheating. This was repeated until, after prolonged heating, the solution remained colourless.

For the FISKE AND SUBBAROW determination it was necessary to use two molybdate solutions, one containing 2.5 % ammonium molybdate in 5 *N* H_2SO_4 and the other containing 2.5 % ammonium molybdate in 3 *N* H_2SO_4. The former was used with aqueous solutions of the sodium phosphate standard and the latter with hydrolysed phospholipids. All colorimetric measurements were made with a Unicam S.P. 500 spectrophotometer.

Thin layer. The purity of fractions from silicic acid columns was checked by thin-layer chromatography on Kieselgel G plates developed in petroleum ether–ether–acetic acid (85:15:1, v/v)[16] and sprayed with 2′,7′-dichlorofluorescein.

Liquid chromatograph

The parameters of the liquid chromatograph were set as shown below.

Oxygen free nitrogen flow rate: through detector 30 ml/min, through cleaner 20 ml/min, and through evaporator 5 ml/min
Hydrogen flow rate for burner: 30 ml/min
Air flow rate for burner: 500 ml/min
Detector temperature: 350°
Cleaner temperature: 600°
Evaporator temperature: 50°
Wire speed: minimum (*ca.* 2 in./sec)
Chart speed: 3 in./h
Amplifier sensitivity: 5 × 10

RESULTS

Recovery of synthetic lipid mixtures

The purity of each component of the mixture was checked quantitatively and by thin-layer chromatography. At least 99 % purity was obtained with all samples except tripalmitin and lecithin. Dipalmitin was found to contain some free fatty acid and this was allowed for in calculating recovery yields.

Neutral lipids. A mixture of cholesteryl palmitate, tripalmitin, diolein, monopalmitin, cholesterol and palmitic acid in petroleum ether (0.5 ml) was eluted from a silicic acid column with increasing concentrations of ether in petroleum ether. The elution chromatogram is shown in Fig. 2a. Table I shows the order of elution of each fraction from the column and its recovery yield.

TABLE I

ELUTION OF A LIPID MIXTURE FROM A SILICIC ACID COLUMN

Eluent (%)		*Component eluted*	*Input concentration* (*μmoles*)	*Recovery* (*μmoles*)	*Yield* (%)
Ether	*Petroleum ether*				
1	99	Cholesteryl palmitate	1.412	1.39	98.5
4	96	Tripalmitin	0.315	0.313	93.4
10	90	Palmitic acid	0.075	0.073	99.4
		Cholesterol	0.168	0.165	97.4
25	75	Diolein	0.506	0.483	98.3
100	—	Monopalmitin	0.913	0.910	99.5

Lecithin. Fig. 2b is the elution pattern of lecithin. Its recovery yield from silicic acid was determined separately by phosphorus determinations before and after elution from the columns because it contained a number of impurities which would have interfered with the neutral fractionation. The recovery yield was 93.5 %. In order to achieve complete elution of lecithin from the column it was necessary to use 100 % methanol as the solvent.

Separation of normal human blood plasma lipids

Lipids were extracted from two identical samples of normal human blood plasma (5 ml) and fractionated by different methods.

Method A. Silicic acid (1 g) was added to the lipid extract in chloroform (3 ml) and shaken for several minutes. The chloroform and silicic acid were separated by centrifugation and the chloroform decanted off. The silicic acid was washed several times with more chloroform and these washings were added to the original chloroform extract which was evaporated to dryness, taken up in petroleum ether (0.5 ml) and fractionated on a silicic acid column in the usual way (Fig. 2c). The phospholipids, adsorbed by silicic acid from the chloroform extract, were eluted with methanol.

Method B. The lipid extract was eluted from a silicic acid column without prior separation of the phospholipids, Fig. 2d.

The results of the two methods are compared in Table II. Tables III gives the order of elution of the lipid classes.

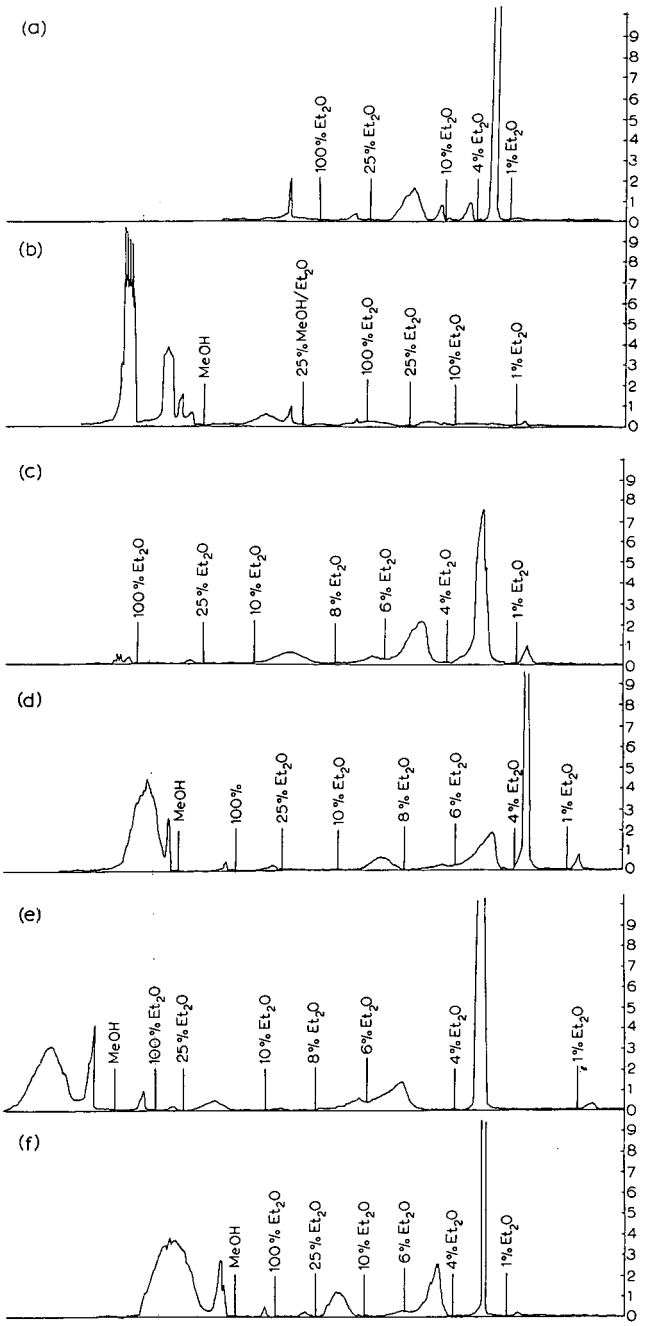

Fig. 2. Liquid chromatograph elution patterns. (a) Separation of synthetic neutral lipid mixture; (b) lecithin; (c) separation of lipids from normal human blood plasma by Method A; (d) separation of lipids from normal human blood plasma by Method B; (e) separation of lipids from normal human blood plasma; (f) separation of lipids from normal human blood serum. 1 cm represents 1 in. on the original chart. Direction of elution is from right to left.

TABLE II

COMPARISON OF NORMAL BLOOD PLASMA LIPIDS

Fraction	*Method A* (*μmoles/ml*)	*Method B* (*μmoles/ml*)
Cholesteryl esters	2.60	2.89
Triglycerides	1.17	1.42
Free fatty acids	0.163	0.166
Free cholesterol	0.889	0.942
Diglycerides	0.110	0.108
Monoglycerides	0.200	0.222
Phospholipids	4.06	5.10

TABLE III

ORDER OF ELUTION OF BLOOD PLASMA LIPIDS FROM A SILICIC ACID COLUMN

Eluent			*Fraction(s) eluted*
Petroleum ether (%)	*Ether* (%)	*Methanol* (%)	
99	1	—	Cholesteryl esters
96	4	—	Triglycerides
94	6	—	FFA/triglycerides
92	8	—	Cholesterol
90	10	—	
75	25	—	Diglycerides
—	100	—	Monoglycerides
—	—	100	Phospholipids

Comparison of normal human blood plasma and serum lipids

Lipid extracts were prepared from plasma and serum samples (5 ml) of normal human blood. The two samples were eluted from a silicic acid column according to Method B, Fig. 2e and f. The results are compared in Table IV. The order of elution was the same as that shown in Table III except that free cholesterol was only eluted from the column when the ether content of the eluent reached 10 %.

TABLE IV

COMPARISON OF NORMAL BLOOD PLASMA AND SERUM LIPIDS FROM THE SAME SOURCE

Fraction	*Plasma* (*μmoles/ml*)	*Serum* (*μmoles/ml*)
Cholesteryl esters	2.59	1.18
Triglycerides	1.51	1.69
Free fatty acids	0.271	0.177
Free cholesterol	1.06	1.08
Diglycerides	0.324	0.273
Monoglycerides	0.301	0.595
Phospholipids	3.98	2.81

DISCUSSION

The recovery yields (Table I) obtained by separating a synthetic mixture of lipids of known concentration demonstrate that the loss of material through monitoring the silicic acid column with a liquid chromatograph is negligible. The time lag between a sample entering the coating block and its detection on the chart recorder is of the order of 5 sec, with the result that the passing of unnecessary volumes of eluent down the column is reduced to a minimum. Secondly, no material is lost in carrying out colorimetric, or other tests, in order to locate the eluted fractions. Fig. 2 demonstrates that 5 ml of plasma is an adequate quantity for the detection of the major lipid classes. With synthetic lipid mixtures complete class resolution was achieved (Fig. 2a). However, in the case of the plasma and serum samples (Fig. 2c–f) some overlap between triglycerides and free fatty acids was observed which was probably due to the presence of unsaturated triglycerides present in blood plasma, as these tend to be eluted after the more saturated compounds[17]. Quantitative estimations of these components were made by carrying out fatty acid determinations, by the DUNCOMBE method[8], before and after hydrolysis. It was found that in some cases free cholesterol was eluted with 8 % ether in petroleum ether whereas at other times 10 % ether was needed. Slight variations in the solvent composition could account for this as solvents were made up immediately before use to avoid loss of one component through evaporation. However, as there is no indication of other lipids being eluted at this solvent polarity it is advisable to elute free cholesterol with 10 % ether in petroleum ether.

In Table II a comparison has been made between two methods of fractionation. In Method A, the phospholipids were separated from the neutral components before fractionation. In Method B they were not. In the latter case this group is retained on the column for the longest period of time. It is apparent that when the two-stage fractionation is carried out there is some loss of material particularly in the phospholipid fraction. This is presumably due to losses of small quantities of solvents and silicic acid during transference from one vessel to another. A similar comparison was made by BATES[18], who made the same observations. The advantage of removing the phospholipids prior to fractionation is that these compounds are subject to autoxidation which could occur through remaining on a silicic acid column for prolonged periods of time unless special precautions are taken[17]. If the phospholipid fraction is required for further investigation it is probably advantageous to adopt Method A, although a certain loss in yield would be involved.

A detailed analysis of blood plasma lipids is frequently of more value if it is accompanied by a protein analysis. Protein analysis is conveniently carried out by cellulose acetate electrophoresis which unfortunately does not always give a clear separation of fibrinogen and β-globulin. For this reason a comparison of plasma and serum lipids was carried out. As is illustrated in Table IV there is some alteration of the lipid pattern during the clotting process which makes analysis of serum less meaningful than that of plasma.

The parameters for the liquid chromatograph cited in the experimental section were found to be satisfactory and were used throughout in the experiments discussed in this paper. The gas flow rates were those recommended by W. G. Pye & Co. Ltd. The other parameters were selected bearing the following points in mind. It is desir-

able to keep the evaporator temperature as low as possible in order to reduce the loss of solute, but at the same time it is important to remove all the solvent. Solvent selection must therefore allow the greatest possible difference between solvent and solute boiling point. The detector temperature must be sufficiently high to allow pyrolysation of the solute but should be at least 200° below that of the cleaner oven so that contaminants, if any, that remain on the wire will not be pyrolysed. Increased sensitivity may be achieved by increasing the wire speed and decreasing the column flow rate. However, neither of these procedures was found to be necessary.

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CHROM. 3898

SOLUBILITY CHROMATOGRAPHY OF SERUM PROTEINS

I. ISOLATION OF THE FIRST COMPONENT OF COMPLEMENT FROM GUINEA PIG SERUM BY SOLUBILITY CHROMATOGRAPHY AT LOW IONIC STRENGTH*

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(Received December 2nd, 1968)

SUMMARY

A method for chromatographic separation of serum proteins on the basis of solubility in buffers of low ionic strength has been developed. Its application to the isolation of the first component of guinea pig complement has led to a two-step process that yielded estimated recoveries of 45 % or more, with an increase in specific activity of at least 250-fold in most runs.

INTRODUCTION

Except for preliminary studies by PORATH[1] and SARGENT AND GRAHAM[2], little use appears to have been made of solubility in the chromatographic separation of proteins. With the advent of ion exchange and gel filtration chromatography, precipitation methods for protein purification have generally been neglected because of their inefficiency. The present series of reports describes several systems in which the advantages of precipitation may be combined with the efficiency of chromatography. The major advantage of precipitation methods is that they make use of conditions which are unfavorable to the action of the proteolytic enzymes present in many crude starting materials such as serum or tissue extracts; substances sensitive to the action of such enzymes may therefore be isolated with greater ease by precipitation than by other methods. Another advantage of precipitation methods over ion exchange is that problems due to the limited exchange capacity of available column packings are avoided, and thus greater quantities of material can be processed at one time.

Solubility chromatography is performed on gel columns of a sufficient degree of cross-linkage to exclude the protein to be purified. Such a gel column is equilibrated with a solution in which some or all of the proteins are insoluble. A zone of precipitated protein is formed on the column, whose packing prevents further movement of the

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precipitate. A front or gradient of solvent is then used to dissolve the proteins selectively. As each protein dissolves, it migrates into the column ahead of the solvent front by virtue of the gel filtration effect, and as a consequence precipitates again, to wait for the solvent front to redissolve it. The result is a counter-current process distributing the different proteins according to their solubilities under the conditions chosen. As there are many parameters that affect the solubility of proteins, the conditions for solubility chromatography can be varied over an extremely broad range.

In view of the above considerations, the use of a gel with a low degree of cross-linkage, *e.g.*, Sephadex G-100, as described by PORATH[1], would be expected to reduce the efficiency of separation since the method depends on the ability of the gel to exclude the protein.

The present report describes the fractionation of serum proteins by solubility chromatography at low ionic strength (μ). The next report in this series will deal with solubility chromatography at high ionic strength, in concentrated ammonium sulfate solutions.

The proteins of interest in this laboratory are the nine components of the complement (C')* system, which together constitute a small fraction of the total serum proteins. The purification of these proteins is fraught with difficulty due to the instability of some of them, and to the fact that C'1, upon activation, destroys C'4 and C'2 (ref. 3). A common first step in many purification schemes[4–7] for the C' components therefore consists in the precipitation of the euglobulin fraction at pH 5.6, $\mu = 0.02$; under these conditions, C'1 is insoluble, as are two of the other C' components, *viz.*, C'3 and C'5 (ref. 7).

The two procedures most widely used to reduce the ionic strength are dilution with distilled water and dialysis. Dilution has the disadvantage that the concentration of the soluble proteins is immediately decreased to a considerable extent, which adds to the difficulty of subsequent purification efforts. The disadvantage of dialysis is that it requires time, during which the precipitate forms slowly and remains in contact with the proteins in the supernatant fluid. The variable yields of C'2 obtained by the method of BORSOS *et al.*[4] can probably be ascribed to inactivation at this point. By solubility chromatography, both of these difficulties can be overcome: On the one hand, dilution of the supernatant fluid is minimized; on the other, the precipitate is separated from the soluble proteins as it forms. FJELLSTRØM[8] has used this method to prepare serum reagents lacking C'1 or C'2 (R1 or R2, respectively[9]), but has apparently not used it to purify C'1.

This report describes the separation of those proteins that are soluble at pH 5.6, $\mu = 0.02$, from those that are insoluble under these conditions on a column of polyacrylamide (Bio-Gel P-10, Bio-Rad Labs., Richmond, Calif.); this gel was chosen to avoid the possibility of reaction of C' components with natural antibodies to dextran

* The components of C' are designated by numbers and are defined in terms of chemical properties and sequence of action on sheep erythrocytes (E) that have previously been treated with antibody (A), forming EA. Thus, C'*j* will denote the j^{th} component of C' according to the definition adopted by the 3rd Complement Workshop[24]. C'*j*a denotes the activated form of C'*j*, which is produced during its reaction with EA, and in some cases, by other manipulations. Intermediates in the sequence by which the C' components act on EA to produce lysis are named EAC'*j*,*i*,*k*,...; the numbers in this expression designate which C' components have reacted with the EA, and imply that C' components that are not listed must be supplied in order to lyse the cells.

which might bind to Sephadex. The average recovery of C'2 in the soluble fraction is 90%. The precipitated C'1 is eluted with a gradient of rising NaCl concentration at pH 5.6; it is recovered in high yield, purified 40-fold. The observation of NELSON *et al.*[7] that C'1 also precipitates at low ionic strength at pH 7.5 is utilized to purify C'1 further from this fraction by solubility chromatography near this pH. These efforts have yielded the surprising result that C'1 exists in two active forms of different solubilities at pH 7.7 (ref. 10).

MATERIALS AND METHODS

Apparatus

The chromatograms shown were obtained by recording the pH, optical densities, and electrical resistance of column effluents automatically. To this end, the effluent was passed successively through a flow-through conductivity cell connected to a recording conductivity bridge (Conductolyser, LKB Instruments), through a micro flow-through cuvette (Pyrocell Mfg. Co., Westwood, N.J.) in a spectrophotometer programmed to record optical densities at three selected wavelengths (Beckman Model DB-G with Programmer), and through a flow-through pH electrode system (Beckman No. 46850) connected to a pH meter (Beckman Expandomatic). Before entering the pH electrode assembly the liquid stream was interrupted by a simple device, shown in Fig. 1, to prevent grounding the glass electrode voltage to the conductivity cell. In spite of this precaution, the pH trace showed regular low-frequency oscillations with an amplitude of about 0.1 pH, which were averaged in the figures shown below. The conductivity cell was maintained at 0.7° in a thermoelectrically cooled bath. The output of the spectrophotometer, which is linear in percent transmission, was applied to a logarithmic amplifier to provide a signal linear in optical density (O.D.). The output signals from all these devices were adapted for input to a six-channel, 10 mV potentiometric recorder (Bristol Multiple-Point Dynamaster),

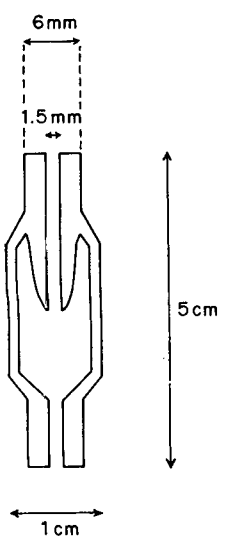

Fig. 1. Design of a stream interruptor for on-stream pH measurement.

equipped with an event marker for the fraction collector. Solutions were pumped through the columns with a peristaltic pump (LKB Instruments) to provide reasonably constant flow rates. All operations were performed in a cold room at 2–4°.

Assay procedures

The isotonic NaCl–Veronal buffer and the hemolytic intermediate EAC'1a,4 were prepared, and C'2 assays were performed, as described by KABAT AND MAYER[11]; the latter procedure was modified as described by HOFFMANN *et al.*[12]. The EAC'1a,4 preparations used in this work had t_{max} values[11] of 10–15 min. The results of C'2 assays were evaluated in accordance with the dose response relation described by HOFFMANN AND MEIER[13].

The intermediate EAC'4 was prepared by a modification[14] of the method of BECKER[15]. EAC'1a,4 were suspended in buffer containing 10 m*M* EDTA to a concentration of *ca.* 10^9 cells/ml and incubated 30 min at 37°. The suspension was centrifuged for 10 min at 1290 × *g* (at r_{max}) and the supernatant fluid was removed by suction. The cells were resuspended as before in buffer containing 10 m*M* EDTA and the incubation, centrifugation, and removal of the supernatant fluid were repeated. The cells were then washed, successively, once in buffer containing 10 m*M* EDTA and twice in buffer containing 0.15 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. Each time the cells were centrifuged, the supernatant fluid was removed, and the cells were resuspended to a concentration of *ca.* 10^9 cells/ml. They were finally suspended in buffer containing Ca^{2+} and Mg^{2+} as above, and stored at 0°.

C'1 assays were performed as described by HOFFMANN *et al.*[12]. The diluted C'1 samples were mixed with the EAC'4 immediately after each dilution was prepared. For assays of C'1 in whole serum, EAC'4 was incubated with the diluted serum sample for 3 h at 37.0° to permit complete activation of the C'1 (ref. 16). The C'2 preparations used in these assays were made by a new procedure to be described in another report; the same results are obtained with the new preparations as were obtained with C'2 lots made according to BORSOS *et al.*[4].

Electrophoretic procedures

Disc electrophoresis was performed in running gels of 7.5 % polyacrylamide at pH 9.5 (ref. 17), with a current of 5 mA per tube in a commercial apparatus (Shandon Scientific Co., Sewicky, Pa.). Since difficulty was encountered in getting the sample gels containing protein to polymerize, the samples (50 µl) were applied in solution containing *ca.* 50 % sucrose, dry polyacrylamide beads (Bio-Gel P-10, 200–400 mesh) being added to each tube to prevent convection[18]. Electrophoresis was continued until the Bromophenol Blue marker dye had just migrated to the end of the column. Stained disc electrophoresis patterns were scanned on a Gilford Model 240 spectrophotometer at 550 nm.

Immunoelectrophoresis was performed in 0.75 % agarose gel in barbital buffer, pH 8.6, $\mu = 0.05$, for 90 min at 5 mA per slide in an apparatus based on SCHEIDEGGER's procedure[19] (Agafor, National Instrument Labs., Rockville, Md.). The patterns were developed with a commercial antiserum to whole guinea pig serum (Immunology, Inc., Glen Ellyn, Ill.). Each sample analyzed by either electrophoretic procedure was adjusted to an O.D. of 3.0 (1 cm light path) at 278 nm. A constant current power supply was used for all electrophoretic procedures (Buchler No. 3-1014).

Chromatography

Column 1. A borosilicate glass column (7.7 cm I.D. × 90 cm) with removable end pieces, fitted with an upward flow adaptor (Glass Engineering Co., Houston, Tex.) was coated with dimethyldichlorosilane and charged with 300 g of polyacrylamide gel, Bio-Gel P-10, 50–150 mesh (both from Bio-Rad Labs., Richmond, Calif.), from which the fine particles had been removed by repeated decantation. The bed so obtained was 46 cm high during operation of the column. Between successive runs, the bed was stirred up completely in an excess of 0.30 *M* NaCl containing 0.01 *M* ethylenediamine tetraacetate (EDTA), pH 8.5; part of the gel was removed from the column temporarily in order to achieve complete resuspension. The gel was allowed to settle and the cloudy supernatant fluid, containing presumably undissolved protein from the previous run, was siphoned off. The column was then equilibrated by upward flow with sodium acetate -acetic acid buffer, pH 5.6, $\mu = 0.020$, at *ca.* 200 ml/h.

Fresh frozen guinea pig serum was either purchased commercially (Immunology, Inc., Glen Ellyn, Ill.) or obtained through the generosity of Dr. KENNETH AMIRAIAN, Division of Laboratories, New York State Department of Health, Albany, N.Y. The commercial serum was separated from blood obtained by cardiac puncture and contained higher concentrations of protein (O.D. at 278 nm) and hemoglobin than the serum from New York, which was separated from blood obtained by exsanguination of guinea pigs under CO_2 anesthesia[20]. This difference affects the chromatographic results as will be discussed in detail below.

Approximately 200 ml of fresh guinea pig serum, which had been stored at −65°, were adjusted to pH 5.5 with 0.15 *M* acetic acid in an ice bath. In this operation, the pH tends to drift back to more alkaline values; adjustment was continued until this drift was less than 0.1 pH in 1 min. This is of cardinal importance to the success of this chromatographic procedure, for too high a pH leads to premature elution of part of the C'1.

The adjusted serum was pumped onto the column at a rate that just prevents a build-up of serum above the top of the bed, followed by a gradient generated by a 9-chamber variable gradient former (Buchler "Varigrad"); the solutions placed in each of the chambers are shown in Table I. The gradient was followed by a 0.30 *M* NaCl solution containing 10 m*M* EDTA, pH 8.5; this step is designed to remove protein that is not soluble anywhere in the gradient, and to destroy traces of C'1 that might be left on the column. A flow rate of 300 ml/h was achieved. Twenty-milliliter fractions were collected by volume (Gilson Medical Electronics fraction collector

TABLE I

GRADIENT PROGRAM FOR COLUMN 1

Each chamber contained 140 ml of the indicated solution, adjusted to pH 5.6 with acetic acid before being made up to volume.

Component	*Concentration in chamber*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Na^+-acetate, *M*	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
NaCl, *M*	0.000	0.018	0.035	0.050	0.180	0.140	0.010	0.400	0.300
$CaCl_2$, m*M*	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Model VL); all tubes beyond the expected position of the excluded (soluble) protein peak contained 1 ml of 1 *M* Tris–HCl buffer*, pH 7.5. In this manner, both the pH and ionic strength of each fraction were raised immediately upon collection to keep the proteins in solution. Electrical resistance was recorded across a flow cell with cell constant 0.704 cm^{-1} (value supplied by manufacturer), and conductivities were computed from this figure and the recorded resistance values. A flow cell with a 5 mm light path was used to record the optical densities.

The fractions containing C'1 were combined and sufficient 3.00 *M* NaCl was added to the pool to increase the NaCl concentration by 0.15 *M*. This was done to compensate for the loss of salts which occurs during subsequent ultrafiltration at high pressure (95 lb./sq. in. of N_2) through a "Diaflo" UM-1 membrane of 3 in. diameter (Amicon Co., Lexington, Mass.); this loss of salts is probably an osmotic pressure effect. Concentration of the 550–700 ml of pooled material to 20–30 ml required 16–23 h; this operation was carried on in the cold room. Recently a PM-30 membrane was substituted for the UM-1 membrane; this reduced the time required for concentration to 10–15 h. An unidentified substance absorbing ultraviolet light was regularly found in the effluents from UM-1 membranes; it had an absorption maximum at 288 nm and a minimum at 260 nm, with $A_{288}/A_{260} = 1.9$. Tests for C'1 activity in the effluents were negative.

Column 2. A borosilicate glass tube of 5.0 cm I.D. with a removable Teflon end piece (Glass Engineering Co., Houston, Tex.) was coated with dimethyldichlorosilane. One hundred grams of polyacrylamide gel (Bio-Gel P-10, 200–400 mesh) were allowed to swell in distilled water. Approximately 25 % of the gel was removed as fine particles in the course of numerous cycles of resuspension, settling, and removal of the supernatant fluid by suction. The remaining gel was packed into the column to produce a bed height of 29 cm under operating conditions. The column was equilibrated with a solution containing 10 m*M* Tris–HCl, pH 7.0 (measured at room temperature) and 0.5 m*M* $CaCl_2$. This produced a pH of 7.7 at 2°, presumably because of a shift in the pK of the buffer.

A linear gradient was formed by means of two identical cylindrical vessels in hydrostatic equilibrium; the mixing chamber initially contained 80 ml of the Tris–HCl–$CaCl_2$ solution with which the column had been equilibrated, while the reservoir contained 80 ml of a solution of 10 m*M* Tris–HCl, pH 7.0 (measured at room temperature), 0.5 m*M* $CaCl_2$, and 0.15 *M* NaCl. This gradient was pumped into the column immediately *before* the concentrated C'1 pool from Column 1. This, in turn, was followed by the starting Tris–HCl–$CaCl_2$ buffer. Flow rates between 20 and 37 ml/h were obtained in the various runs on this column for which results are presented in this report. Fractions of 5 ml were collected by volume into 0.25 ml of 1.00 *M* NaCl (Beckman/Spinco fraction collector). Recording conditions were the same as for Column 1, except that a cuvette with a 10 mm light path was used to record the O.D.

RESULTS

Solubility chromatography at pH 5.6

Fig. 2 shows a chromatogram obtained with Column 1. Optical densities were recorded at 278, 360, and 412 nm. The first peak, representing proteins soluble at

* Tris = Tris-(hydroxymethyl)-aminomethane.

pH 5.6, $\mu = 0.02$, contains heme proteins whose O.D. at 412 nm is greater than that at 360 nm. The effluent emerging later in the run is turbid; this is reflected in an O.D. at 360 nm which is greater than that at 412 nm. All fractions in the early protein peak with an O.D. at 278 nm greater than 2.0 were pooled on the basis of the recorded O.D.; they represent *ca.* 70 % of the applied protein. This pooled material frequently becomes slightly turbid at 0°; it is therefore centrifuged 1 h at 10,000 × *g* at 0°. It is then processed for C'2 production as will be described in the next report in this series.

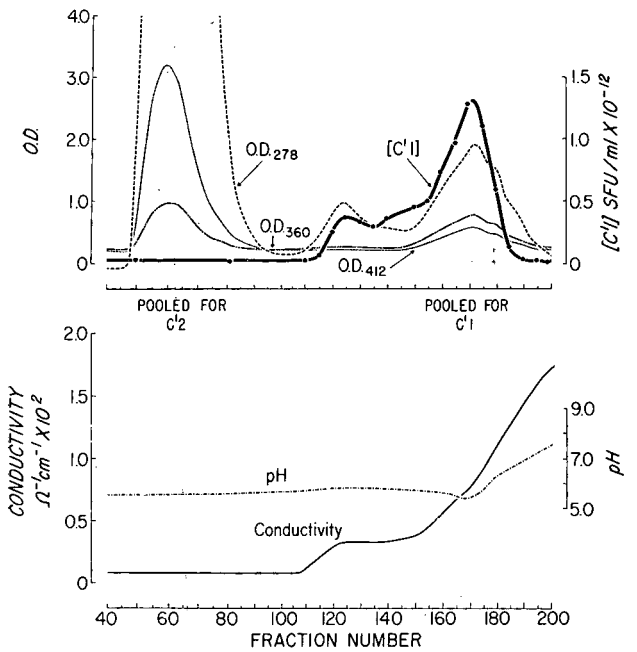

Fig. 2. Solubility chromatogram of 200 ml of whole serum at pH 5.6 and low ionic strength. Fractions: 20 ml. O.D. values are corrected to a 1 cm light path.

Some protein, containing a small amount of the C'1, is eluted in the fractions (No. 110 to 135) which contain the salts present in the applied serum. The size of the column in relation to the volume of serum applied is purposely chosen to be considerably larger than would be needed for a simple buffer change in order to allow these salts to be diluted by diffusion. If the pH adjustment of the serum before application to the column is not performed properly as described above, the pH of these fractions rises above 6.0 and considerable C'1 is eluted in this peak. Most of the C'1 emerges later in the gradient.

The chromatogram shown in Fig. 2 is representative of those obtained with commercial guinea pig serum. Chromatograms for serum from the New York State Department of Health are different in two respects: (i) The single, skewed peak of material absorbing at 412 nm with a maximum at fraction 60 in Fig. 2 is replaced by two well-defined peaks with O.D. maxima below 1.0, the first of which occurs at fraction 60, the second at fraction 80. The second peak is apparently only partially soluble at pH 5.6, $\mu = 0.020$, and thus emerges later than the soluble proteins. It

differs further from the first peak in its absorption spectrum, having a much higher ratio of $O.D._{412}/O.D._{360}$. Additional chromatographic work, incidental to studies to be reported elsewhere, has shown that these two substances also differ in solubility in concentrated ammonium sulfate solutions and in molecular size. (ii) The $O.D._{278}$ of material emerging in the gradient continues to rise past the maximum shown in Fig. 2 at fraction 170 and reaches a peak of 2.6 at fraction 185, after the C'1 is eluted. Since in later runs, fractions for the C'1 pool were selected on the basis of the O.D. pattern rather than after assay, this fact led to an error in pooling fractions from Run XXXV, the first made on serum from the New York State Department of Health; this error lowered both the apparent yield and recovery values (*cf.* Table II).

The extent of purification was estimated on the basis of absorbance, A, at 278 nm measured in a Zeiss PMQ-II spectrophotometer. Since part of the O.D. of a turbid sample at this wavelength (λ) is due to light scattering, use was made of the fact that in spectral regions outside absorption bands, all of the O.D. is due to light scattering and is proportional to λ^{-4}. Spectra in the visible range to 600 nm were therefore taken of all turbid samples; the O.D. values were plotted on log-log paper against λ and the linear portion of each such graph was extrapolated to 278 nm to estimate the light scattering contribution to the O.D. at that wavelength; usually, this represented about 60% of the O.D. for the pooled C'1 from Column 1. The remainder of the O.D. after subtraction of the light scattering contribution was taken as A_{278}.

The results of 11 successive runs are shown in Table II. The soluble fraction shows generally high recoveries of C'2; the only notable exception was Run XXXIII, in which attempt was made to remove turbid material from the serum after adjustment to pH 5.5 by high-speed centrifugation; this led to both mechanical loss and inactivation, and the practice was abandoned. No explanation is available for the low recovery in Run XXXVII. The amount of C'1 remaining in this fraction is usually

TABLE II

RESULTS OF SOLUBILITY CHROMATOGRAPHY AT pH 5.6

Run No.	*Soluble fraction*			*C'1 pool*	
	C'2		*C'1*		
	% recovery	*Purification*	*% remaining*	*% recovery*[a]	*Purification*[a]
XXVIII	90	1.3×	0.8	460	154×
XXIX	99	1.8×	<0.05	360	105×
XXX	97	1.4×	0.042	124	47×
XXXI	103	1.3×	0.33	104	41×
XXXII	92[b]	1.2×	0.04	139	41×
XXXIII	82[b]	1.1×	0.002	82	35×
XXXIV	90	1.2×	<0.02	107	53×
XXXV	93	1.2×	<0.02	44[c]	19×[c]
XXXVI	88	1.2×	0.05	95	43×
XXXVII	77	1.1×	<0.004	263	104×
XXXVIII	93	1.3×	<0.02	238	86×

[a] Assays of C'1 pool and starting material not strictly comparable; *cf.* text.

[b] Corrected for mechanical losses of 18% in XXXII and 45% in XXXIII, due to fraction collector failures.

[c] Mechanical loss; see text.

well below 1 % of input; in a number of runs, it was undectable. The results on apparent recovery and purification of C'1 in the C'1 pool vary considerably. This variation can probably be ascribed to uncertainties in the C'1 assay for whole serum, which derive from the facts that an inhibitor of C'1 is present in whole serum, and that the dose response of C'1 in serum is non-linear[21], in contrast to that of isolated C'1. All that can be said is that the yields are substantial; if the relative activities of whole serum and pooled C'1 are adjusted to give a yield of 100%, the extent of purification averages 40-fold, with individual values between 29- and 50-fold.

The ultrafiltration step yields consistently high recoveries, often exceeding 100 % of the activity contained in the pooled C'1. This is probably due to the reversible, concentration-dependent dissociation of C'1 into inactive subunits described by COLTEN *et al.*[22]. If the pooled C'1 is in a partially dissociated state, the 20-fold increase in concentration during ultrafiltration should reverse that dissociation.

The total time required for a normal run on this column is 11 h; elution of the soluble fraction is complete in 4½ h, that of C'1 in 10 h.

A typical chromatogram obtained with Column 2 is shown in Fig. 3. Most of the protein emerges with the void volume of the column. This fraction always contains some C'1 activity, which has been labeled Type A, and represents a form of C'1 which is soluble at $\mu = 0.0105$, pH 7.7. If the C'1 from Column 1 is concentrated and applied to Column 2 without delay, most of the C'1 emerges in the gradient; this C'1 fraction has been designated as Type B. The conductivity corresponding to the maximum in the C'1 activity curve shows some variation from run to run; this may be due to variations in volume and salt concentration in the applied sample. In most runs, the

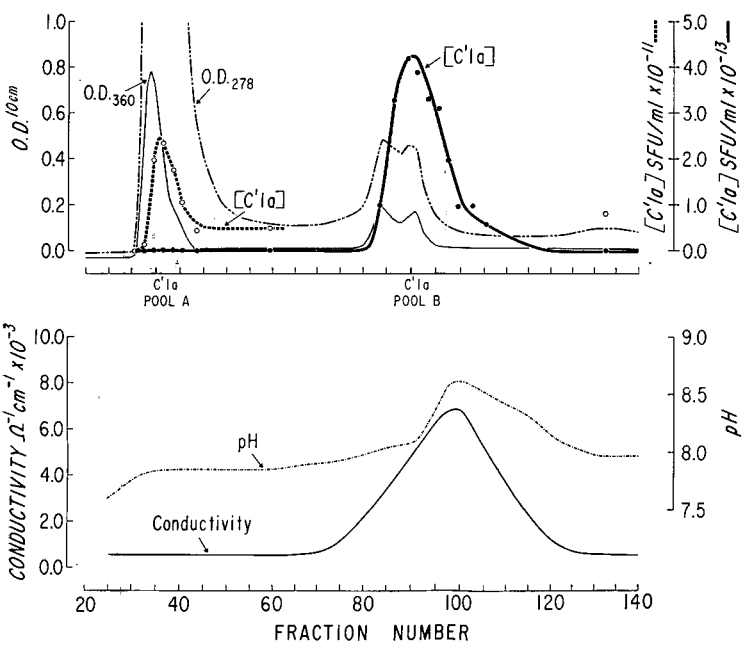

Fig. 3. Solubility chromatogram of pooled, concentrated C'1 from Column 1 (Fig. 2) at pH 7.7 and low ionic strength. Fractions: 5 ml.

conductivity at that point in the leading edge of the C'1 zone where the C'1 concentration attains half its maximal value has been fairly reproducible; it is equivalent to an NaCl concentration of 0.076 ± 0.006 *M*. The 60 ml of effluent following this point in the conductivity curve contain the bulk of the C'1. Occasionally, the C'1 emerges earlier in the gradient; no explanation is available for this fact.

The chromatographic behavior of the two types of C'1 is reproducible when each is chromatographed separately under the same conditions[10]. The present findings differ from those described in the earlier report[10] in that at that time, most of the C'1 recovered was Type A; this is due to instability of Type B C'1 in the concentrated preparation obtained from Column 1; thus, if application to Column 2 is delayed, loss of Type B occurs. This loss is not accompanied by a corresponding increase in Type A[21].

A preliminary report on the different biological properties of the two types of C'1 has appeared[23]. Type B has all the properties usually ascribed to C'1a; Type A lacks some of these properties and closely resembles the subunit, C'1s, of human C'1 as described by NAGAKI AND STROUD[24]. No Type A C'1 is recovered when whole guinea pig serum is subjected to solubility chromatography at pH 7.7; what causes its formation, presumably during operation of Column 1, is not known. Partially purified guinea pig C'1, obtained by other methods[6], can also be separated into Types A and B by the procedure described here[25]. A detailed report on the biological and physical properties of the two types of C'1 is in preparation.

Experience with eight successive runs on Column 2 is summarized in Table III. The recoveries of Type A are quite variable and tend to be lower for Runs XXXV and up, *i.e.*, for those performed with serum obtained from the New York State Department of Health. Purification of Type A is less than 1-fold because most of the protein contained in the concentrated C'1 from Column 1 emerges with this fraction, which contains only a small portion of the applied C'1. For most runs, the recovery of Type B ranges between 40 and 60 % of input, at 6- to 10-fold purification. No explanation is available for the anomalously high recovery in Run XXXII, nor for the low recovery in Run XXXV. The latter has been confirmed by repeated assay; the only unusual feature in this run was that C'1 application to the column began after 84 % of the gradient had entered the column, instead of the usual 93 %. The 101 % recovery in Run XXXVI may reflect variation in assay results between lots of EAC'4 of different ages. The normal running time for this column is 24–36 h.

TABLE III

RESULTS OF SOLUBILITY CHROMATOGRAPHY AT pH 7.7

Run No.	*Peak A*		*Peak B*	
	% *Recovery*[a]	*Purification*[a]	% *Recovery*[a]	*Purification*[a]
XXXI	5.8	<1×	43	2.4×
XXXII	13	<1×	309	6.8×
XXXIII	2.4	<1×	57	8.0×
XXXIV	3.0	<1×	45	9.8×
XXXV	0.16	<1×	9.6	6.0×
XXXVI	0.46	<1×	101	9.2×
XXXVII	0.51	<1×	82	13.0×
XXXVIII	0.17	<1×	41	5.2×

[a] Recoveries and purification both relative to material applied to the column.

Results for the combined operation of the two chromatographic procedures are shown in Table IV as recoveries and purification ratios with respect to whole serum. These figures are subject to the difficulties arising from assays on whole serum, discussed in connection with the data in Table II. Subject to qualifications arising from this source, it would appear that the procedures described will yield at least 30 to 50 % recovery of Type B C'1 at 250- to 300-fold purification.

TABLE IV

OVER-ALL RESULTS FOR BOTH STEPS: TYPE B C'1

Run No.	% *Recovery*	*Purification*
XXXI	45	98×
XXXII	430	280×
XXXIII	48	280×
XXXIV	36	250×
XXXV	6.7[a]	170×
XXXVI	174	710×
XXXVII	203	1350×
XXXVIII	77	690×

[a] In part reflects mechanical loss; see text.

The results of electrophoretic analyses of the various fractions obtained are shown in Figs. 4 and 5, the former representing tracings of scans of disc electrophoretic patterns, the latter reproducing the stained immunoelectrophoresis slides. They show that qualitative changes in composition have occurred as a result of these chromatographic procedures; this is consistent with the changes in specific activity of C'1 shown in the tables.

Comparison of the disc electrophoresis patterns for whole serum (A) and the fraction soluble at pH 5.6, $\mu = 0.02$ (B) shows two major differences: The band just after albumin, and the sharp band which barely enters the separating gel, which are seen in the pattern for whole serum, are absent in the soluble fraction. Analyses at lower protein concentration show that a small amount of the faster protein does, in fact, remain in the soluble fraction, but is obscured here because of excessive spreading of the albumin band. These two bands both appear at a considerable increase in relative concentration in the pooled C'1 from Column 1 (pattern C); however, the faster of these two bands did not penetrate the separating gel as deeply as did its apparent counterpart in pattern A, and this identification must remain tentative. The pattern for C'1 Pool A from Column 2 (D) does not differ appreciably from that of the parent fraction (C), except that the faster of the two main peaks in pattern D moved even more slowly than its counterpart in pattern C. All of the samples were subjected to electrophoresis simultaneously, and the observed mobility differences are greater than those found in replicate patterns for whole serum. The pattern for C'1 Pool B from Column 2 (E) shows one major band of very low mobility, consistent with the high molecular weight of C'1 (ref. 26) ,which would prevent this molecule from penetrating very far in the 7.5 % polyacrylamide gel.

The immunoelectrophoretic patterns generally confirm the observations made by disc electrophoresis. Patterns A and B, for whole serum and the fraction soluble

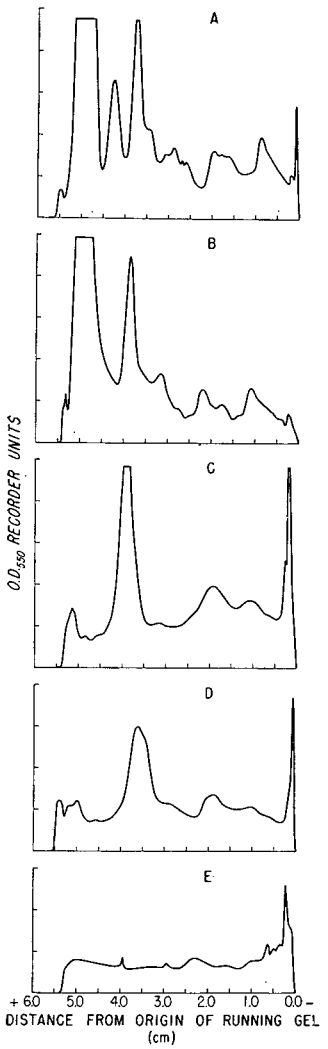

Fig. 4. Disc electrophoresis patterns of whole serum and fractions obtained by solubility chromatography. Scan A: whole serum; scan B: soluble fraction from Column 1; scan C: pooled C'1 from Column 1; scan D: C'1 Type A from Column 2; scan E: C'1 Type B from Column 2, examined at one-half the protein concentration of the other samples.

at pH 5.6, $\mu = 0.02$, respectively, are very similar, as are patterns C and D, for pooled C'1 from Column 1 and C'1 Pool A from Column 2, respectively. A notable exception is the prominent band with a mobility slightly less than that of albumin, which is seen in the disc electrophoretic patterns for whole serum, pooled C'1 from Column 1, and C'1 Pool A from Column 2; no arc corresponding to this protein is found in the immunoelectrophoresis patterns. This may be due to the absence of an antibody to this constituent in the antiserum used. The patterns for pooled C'1 from Column 1 (C) and for C'1 Pool A from Column 2 (D) bear little resemblance to the pattern for whole

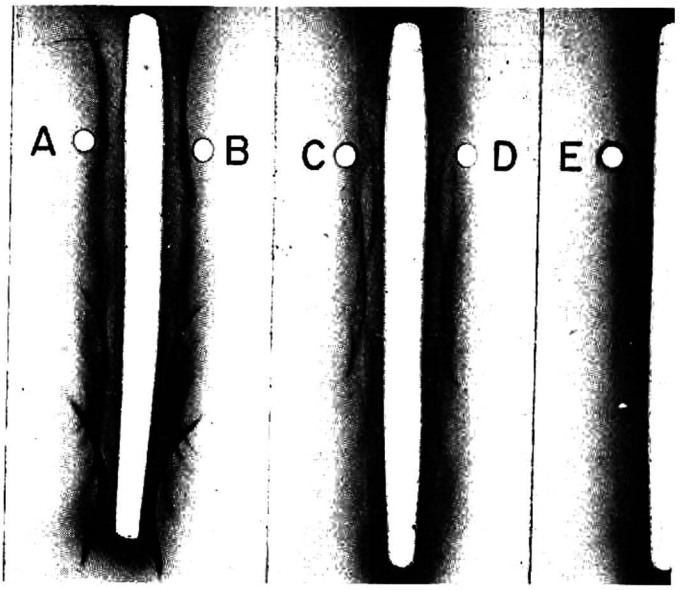

Fig. 5. Immunoelectrophoresis patterns of fractions obtained by solubility chromatography. Pattern A: whole serum; pattern B: soluble fraction from Column 1; pattern C: pooled C'1 from Column 1; pattern D: C'1 Type A from Column 2; pattern E: C'1 Type B from Column 2, examined at one-half the protein concentration of the other samples. The anode is at the bottom.

serum (A); this is not surprising in view of the fact that the pooled C'1 contains only a small fraction (2–3%) of the total serum proteins; because of their low concentration, these proteins are not detected in the pattern for whole serum. The pattern for C'1 Pool B from Column 2 shows three or four arcs, indicating that this preparation is still not homogeneous. The antigen well in this pattern is surrounded by precipitate, which trails off toward the anode; a very faint ring of similar appearance also surrounds the antigen well of pattern C, but not that of pattern D. This precipitate may be due to C'1, which should be virtually insoluble at the ionic strength (0.05) at which the immunoelectrophoretic analysis was carried out.

DISCUSSION

The data presented show that solubility chromatography is a useful method of purification for a protein which is insoluble at low ionic strength. The expected advantages of the method, discussed in the Introduction, appear to have been realized.

There are three ways in which a protein might be applied to the column for solubility chromatography: (i) in precipitated form, preceding the gradient; (ii) in solution, preceding the gradient; and (iii) in solution, after the gradient. The first of these was not suited to the problem at hand, but was studied as an option in the development of solubility chromatography in concentrated ammonium sulfate solutions, which will be considered in the next report. Method (ii) was applied to Column 1; it requires special care in the choice of column volume, for in this case the

salt solution in which the protein was dissolved at the outset will precede the eluting gradient down the column. Unless it is suitably diluted by diffusion during its passage down the column, it will dissolve the precipitated protein ahead of the gradient. In spite of this disadvantage, method (ii) is useful in situations where immediate separation of the precipitate from the soluble fraction is desired, and fractionation of the precipitated proteins is of secondary importance. Method (iii) was used for Column 2; it will be the method of choice for most applications. In this case, the column should be designed so that its internal volume, *i.e.*, that from which macromolecules are excluded, is about twice the volume of the gradient one intends to use; this permits the soluble fraction of proteins to migrate through the entire gradient and to emerge ahead of it, while individual insoluble proteins precipitate in zones in the gradient where their solubilities are exceeded.

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CHROM. 3899

SOLUBILITY CHROMATOGRAPHY OF SERUM PROTEINS

II. PARTIAL PURIFICATION OF THE SECOND COMPONENT OF GUINEA PIG COMPLEMENT BY SOLUBILITY CHROMATOGRAPHY IN CONCENTRATED AMMONIUM SULFATE SOLUTIONS*

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SUMMARY

A method for the chromatographic separation of serum proteins by solubility in concentrated ammonium sulfate solutions has been developed. The fraction of serum proteins which is soluble in 2.20 *M* $(NH_4)_2SO_4$ at pH 6.0 can be resolved into six distinct components detectable by absorbance at 278 or 412 nm. The method is useful for the preliminary purification of the second component of complement from guinea pig serum; recoveries ranged from 23 to 73 %, with a ten-fold increase in specific activity. The effects of a number of variables on separation efficiency are described.

INTRODUCTION

The preceding report in this series[1] describes a method for chromatographic separation of serum proteins by solubility in buffers of low ionic strength. Here we describe the extension of solubility chromatography to concentrated solutions of ammonium sulfate. The method is applied to the preliminary purification of the second component of guinea pig complement, C'2**, starting with the serum fraction which is soluble at ionic strength, $\mu = 0.020$, pH 5.6.

MATERIALS AND METHODS

Apparatus

The automatic system for analyzing effluents from chromatography columns, described in the preceding report[1], was used. But recordings of pH could not be obtained because the saturated KCl in the reference electrode junction precipitated with the $(NH_4)_2SO_4$ in the solution being analyzed. (This phenomenon does not

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** The abbreviations used are the same as those given in the preceding report[1].

preclude quick pH measurements on $(NH_4)_2SO_4$ solutions with the usual dipping electrodes, but manifests itself after prolonged contact.) The flow cell used for conductivity measurements had a cell constant of 122.6 cm^{-1}. Since the conductivity of concentrated $(NH_4)_2SO_4$ solutions increases with concentration in a non-linear manner, a calibration plot was used to convert conductivity to ammonium sulfate concentration.

Assay procedures

The methods used were described previously[1]. The results of C'2 assays were expressed in terms of site-forming units (SFU)[2] per ml; day to day variations in assay sensitivity were estimated by calibration against a standard lot of C'2. This lot, in turn, had been standardized by comparison to two samples of C'2 containing a known number of C'2 units per ml as defined by MAYER AND MILLER[3]; these samples were generously provided by Dr. MANFRED M. MAYER, Department of Microbiology, The Johns Hopkins University School of Medicine.

Electrophoresis

The electrophoretic procedures described in the preceding report[1] were followed here, with the exception that samples for disc electrophoresis were diluted to an optical density of 1.5 at 278 nm (1 cm light path).

Chromatography

We used a borosilicate glass column, 10.2 cm I.D. × 90 cm long, fitted with removable end pieces and an upward flow adaptor (Glass Engineering Co., Houston, Tex.). The column was coated with dimethyldichlorosilane (Bio-Rad Labs., Richmond, Calif.) and charged with 650 g of Sephadex G-50, particle size 20 to 80 μ. This produced a final bed height of 69 cm under operating conditions. The gel was equilibrated by upward flow with 2.7 M $(NH_4)_2SO_4$, pH 6.0 (Mann Research Labs., Special Enzyme Grade); attempts at equilibration by downward flow were complicated by convective disturbances due to density differences between concentrated $(NH_4)_2SO_4$ solutions. Between successive runs, the bed of this column was completely stirred up to prevent excessive packing, which reduces the flow rate; part of the gel was temporarily removed from the column to accomplish this. The operation was usually performed before equilibration. The exclusion and total volumes of the column were determined with Blue Dextran (Pharmacia Fine Chemicals, Piscataway, N.J.) and $(NH_4)_2SO_4$, respectively; the difference between the two volumes was 4.1 l, and was assumed to remain constant in spite of small variations in total bed volume due to differences in packing from run to run.

Chromatographic separations were performed by downward flow. First, a non-linear 1.8 l gradient of $(NH_4)_2SO_4$ concentrations decreasing from 2.70 M to 1.80 M was applied. Each chamber of the gradient former contained 200 ml of the solution indicated in Table I; all solutions were adjusted to pH 6.0.

The protein fraction soluble at pH 5.6, ionic strength $\mu = 0.020$, was obtained from 200 ml of guinea pig serum by solubility chromatography[1]. The total amount of protein to be applied to the Sephadex column was reduced by a preliminary $(NH_4)_2SO_4$ precipitation process. Solid ε-amino-n-caproic acid (EACA, Calbiochem, Los Angeles, Calif.) was added to the protein solution to a concentration of 0.1 M to inhibit serum

TABLE I

AMMONIUM SULFATE GRADIENT SOLUTIONS

	Chamber								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
$[(NH_4)_2SO_4]$, *M*	2.70	2.65	2.55	2.50	2.45	2.40	2.35	2.20	1.80
[EACA[a]], *M*	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

[a] ε-Amino-*n*-caproic acid.

proteases; then, solid $(NH_4)_2SO_4$ was added to a concentration of 2.20 *M*, and the pH was adjusted to 6.0. The amount of $(NH_4)_2SO_4$ required was determined on the basis of density measurements on a series of $(NH_4)_2SO_4$ solutions. All additions were performed in an ice-water bath. The solution was stirred at 0° for another 30 min and then centrifuged at —5° for 20 min at 10,000 × *g*. The resulting precipitate generally contained less than 10% of the C'2 activity present in the starting material. The proteins in the supernatant fluid were precipitated by addition of further $(NH_4)_2SO_4$ to a concentration of 3.50 *M*. The mixture was adjusted to pH 6.0 and stirred for 30 min at 0°, and then centrifuged at —5° for 1 h at 10,000 × *g*. The resulting supernatant fluid was checked for protein by measuring the optical density (O.D.) at 278 nm of a 1:2 dilution in H_2O; the O.D. values obtained were less than 0.04. The precipitate was resuspended in a minimal amount of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0.

These precipitation procedures were always carried out immediately after the protein emerged from the pH 5.6, $\mu = 0.020$ solubility chromatography column. The precipitated crude C'2 fraction was stored at —65° until use; under these conditions, there was no loss of activity for several months.

For application to the column, the suspension of precipitate was carefully thawed, redissolved in an equal volume of ice-cold 0.2 *M* EACA, and centrifuged at —5° for 30 min at 10,000 × *g*. The supernatant fluid was pumped into the column immediately after the gradient, followed by 1.80 *M* $(NH_4)_2SO_4$, pH 6.0.

Flow rates close to, but not exceeding, 200 ml/h were maintained; higher flow rates led to artefacts. The first 2.7 l of effluent contained no protein and were collected into a graduate cylinder; then, 20 ml fractions were collected into 2 ml of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0, per tube. This procedure was designed to minimize degradation of the C'2 in the collected fractions by precipitating most of the protein.

After assay, the relevant fractions were pooled and the conductivity of the solution was measured. The $(NH_4)_2SO_4$ concentration corresponding to this conductivity was determined from a calibration chart, and the amount of solid $(NH_4)_2SO_4$ to be added to the solution to adjust its concentration to 3.50 *M* was calculated from the density data referred to earlier. The addition of this $(NH_4)_2SO_4$ was preceded by sufficient solid EACA to increase its concentration by 0.10 *M*. The pH was adjusted to 6.0 and the mixture was stirred at 0° for 30 min, followed by centrifugation at —5° for 30 min at 10,000 × *g*. The supernatant fluid was checked for protein concentration as above, and the precipitate was resuspended in a minimal volume of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0, and stored at —65°.

The normal running time for this column is 48 h from the time of application of the protein.

RESULTS

A typical chromatogram is shown in Fig. 1. Four peaks—three major and one minor—can be identified by O.D. at 278 nm. The O.D. at 412 nm, for proteins absorbing in the Soret band, reveals two additional peaks which do not coincide with any of the 278 nm peaks. The O.D. curve at 360 nm shows that both 412 nm peaks represent absorbance rather than just turbidity, for in that case the O.D. at 360 nm would exceed that at 412 nm. The small spike in O.D. at 278 nm, centered over fraction 137, is seen in some, but not all, chromatograms.

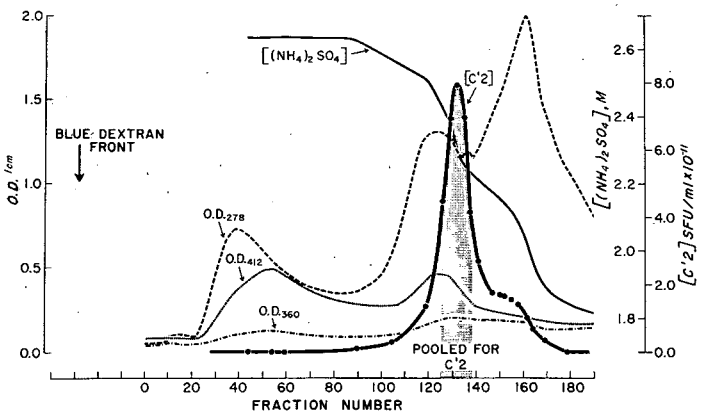

Fig. 1. Solubility chromatography of the serum protein fraction soluble in 2.20 M $(NH_4)_2SO_4$, in concentrated $(NH_4)_2SO_4$ solutions. Fractions: 20 ml. Calibration of assay system shows 1.84×10^8 SFU/unit of C'2.

None of the proteins emerges in the exclusion volume, even though several peaks occur in a region of the chromatogram where the $(NH_4)_2SO_4$ concentration is a constant 2.7 M. (The difference between 2.7 M and the indicated value probably arises from a combination of instrument and calibration errors.) This indicated that none of these proteins is completely soluble in 2.7 M $(NH_4)_2SO_4$, and that they are retarded on the column to different degrees depending on their solubilities in this medium.

The C'2 emerges in a narrow zone with some tailing; the amount of tailing is usually less than shown here. No peak in O.D. at 278 nm is associated with the C'2 maximum, but this is not surprising because of the crude nature of the starting material. The elution position of the C'2 is highly reproducible: In four of the six runs for which results are presented in Table II, the maximum in C'2 concentration occurred at 2.28 M $(NH_4)_2SO_4$; in the remaining two runs it occurred at 2.19 M and 2.37 M $(NH_4)_2SO_4$, but the position of the C'2 band relative to the $O.D._{278}$ pattern was unchanged.

The recoveries and degrees of purification for six representative runs are presented in Table II. Purification was calculated as increase in specific activity on the basis of measurements of the O.D. at 278 nm of samples diluted in H_2O so as to dissolve any precipitate present. The recoveries vary from run to run; this probably

TABLE II

RESULTS OF SOLUBILITY CHROMATOGRAPHY IN CONCENTRATED $(NH_4)_2SO_4$

Run no.	*2.2 to 3.5 M $(NH_4)_2SO_4$ precipitate*		*Pooled fractions*			
			Based on input		*Over-all*	
	% *Yield*[a]	*Purification*[a]	% *Yield*	*Purification*	% *Yield*	*Purification*
XXXIII-6	63[b]	1.6 ×	92	5.1 ×	73	11.5 ×
XXXIV-3	73	2.0 ×	26	3.7 ×	28	10.9 ×
XXXV-3	61	1.7 ×	41	4.5 ×	44	12.1 ×
XXXVI-3[c]	68	1.7 ×	38	3.1 ×	35	7.2 ×
XXXVII-3	40	3.2 ×	73	7.5 ×	23	10.5 ×
XXXVIII-3	79	2.0 ×	46	5.4 ×	34	10.4 ×

[a] Based on whole serum; these figures are derived from assays of samples of resuspended precipitate, and are thus less reliable than usual.

[b] After correction for 45.9 % mechanical loss on 1st column[1].

[c] A different gradient was used in this run; *cf.* text.

reflects the extreme lability of the protein being purified. An example of this lability is given by NELSON *et al.*[4], who state that ion exchange chromatography of the serum protein fraction soluble at pH 7.5, $\mu = 0.04$, on diethylaminoethyl cellulose leads to "low" C'2 recovery. The exceptionally low yield of C'2 in run XXXVII-3 can probably be ascribed to a technical error which occurred in the initial precipitation procedure. In run XXXVI-3, a different gradient from the one described in Table I was used in an attempt to improve resolution; this gradient was shallower in the region where C'2 elutes. Evidently, this results in less purification than is obtained with the gradient shown in Table I.

The disc electrophoresis patterns in Fig. 2 give a qualitative picture of the progress of purification through the procedures described. By comparison with pattern A (proteins soluble at pH 5.6, $\mu = 0.020$), pattern B shows that precipitation with 2.20 *M* $(NH_4)_2SO_4$ removes most of the β- and γ-globulins, and results in a concurrent enrichment in the albumin and α-globulin regions. Solubility chromatography in $(NH_4)_2SO_4$ (pattern C) eliminates the remaining protein of slow γ mobility at 0.5 cm and markedly alters the composition of the α-globulin region (2.5 to 3.5 cm), without appreciably affecting the amounts of albumin and of residual β-globulin (2.0 cm) present.

Examination of the immunoelectrophoresis patterns in Fig. 3 leads to essentially the same conclusions. Pattern C (for the 2.20 *M* $(NH_4)_2SO_4$ supernatant fluid) is remarkable for the absence of an arc corresponding to the prominent slow γ-globulin seen in the corresponding disc electrophoresis pattern; either the antiserum used contains no antibody against this protein, or the molecular weight of this constituent is high enough to cause an appreciable reduction in its electrophoretic mobility in 7.5 % polyacrylamide gel because of exclusion effects.

DISCUSSION

The purpose of the present study was to develop a new class of chromatographic procedures for proteins, based on differences in solubility, which would be particularly

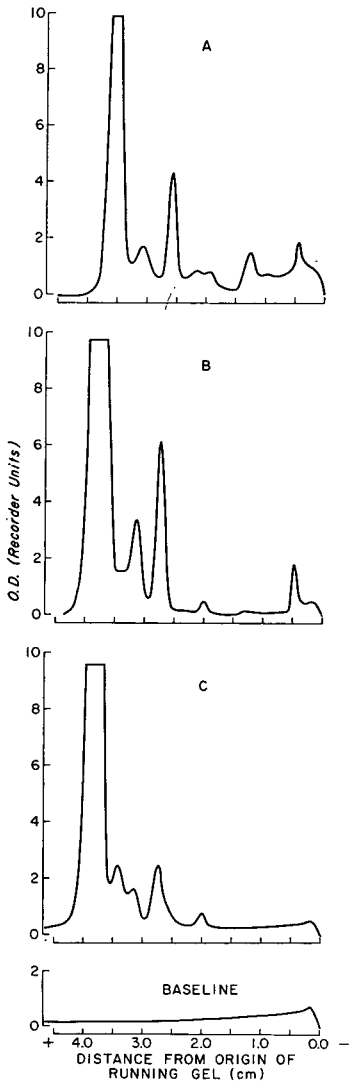

Fig. 2. Disc electrophoresis patterns of partially purified C'2 preparations. Pattern A: serum proteins soluble at pH 5.6, μ = 0.020 (starting material). Pattern B: fraction soluble in 2.20 *M* $(NH_4)_2SO_4$, pH 6.0. Pattern C: concentrated C'2 fraction. Baseline: pattern obtained from a gel without protein.

suited for application to proteins that are labile in solution, and to situations where it is desirable to process substantial quantities of protein. The preliminary purification of C'2 from guinea pig serum, in which it is present in very small amounts, is a problem that presents both of these difficulties.

The first chromatographic procedure for the preparation of partially purified C'2 was developed by BORSOS *et al.*[5] in 1961. The largest amount of serum that can be handled conveniently by this method is 40 ml, and the required time is one day. The

product has the great advantage of being stable. The increase in specific activity over serum is less than 10-fold, but recoveries have been rather variable in our hands, indicating that the method is unduly sensitive to minor variations in technique. C'2 preparations made by this method are free of C'1 and C'4 (ref. 5), but some of the other C' components may be present in substantial amounts.

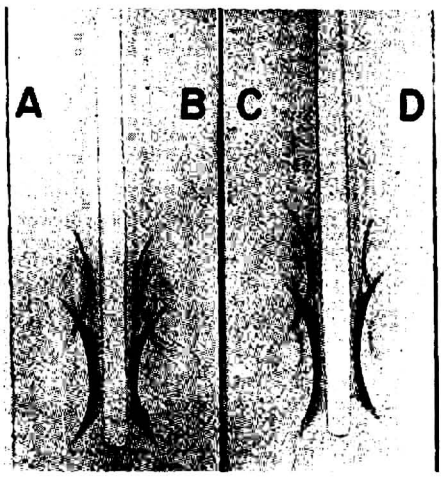

Fig. 3. Immunoelectrophoresis patterns of serum and fractions. Pattern A: whole guinea pig serum. Pattern B: serum proteins soluble at pH 5.6, μ = 0.020. Pattern C: fraction soluble in 2.20 M $(NH_4)_2SO_4$, pH 6.0. Pattern D: concentrated C'2 fraction.

NELSON *et al.*[4] have described a method for purification of C'2 by two successive steps of ion exchange chromatography on carboxymethyl cellulose at pH 5.0 and 5.5, respectively. This procedure requires several days, and the amount of serum handled in one preparation is 30 ml. The product is free of other C' components, but the small amounts of material obtained have not permitted application of criteria of chemical purity. In the only run for which NELSON *et al.* supply information on recovery, it appears to have been about 75%; inactivation was apparently circumvented by working at rather high dilutions.

Recently, POLLEY AND MUELLER-EBERHARD[6] have reported the isolation of a highly purified fraction with C'2 activity from human serum by a sequence of steps consisting of ion exchange chromatography on carboxymethyl cellulose, electrophoresis on Pevikon gel, and chromatography on hydroxyapatite. In their procedure, 700–800 ml of serum are handled at one time. In the two runs for which their report provides details, recovery in the first step was 29 and 32%, with an 8- to 18-fold increase in specific activity. In order to achieve these results, POLLEY AND MUELLER-EBERHARD found it necessary to treat their starting material with diisopropyl fluorophosphate (DFP) to inactivate serum proteases. Their report provides no information on contamination with other C' components.

In our study, we have developed a method by which 200 ml of serum can be processed in a total of three days of column operation (not counting preparation of solutions, etc.) to yield a partially purified product, usually in 28 to 44% yield at 10- to 12-fold purification. The C'2 was protected from enzymatic inactivation by the

presence of 0.1 *M* EACA; it would be impossible to use this inhibitor at this concentration in ion exchange chromatography, since such a high level of zwitterions would interfere with the ion exchange process. The ability to use this substance, rather than the highly toxic and volatile DFP, to protect the C'2 constitutes one of the advantages of this method. We have not tried to process more than 200 ml of serum at a time, but there appears to be no reason why larger amounts could not be handled on the same column; however, one might anticipate some reduction in flow rate as a result of the presence of larger amounts of precipitate on the column. Since the product obtained is still far from pure, we have not looked for the presence of other C' components; on the basis of the $(NH_4)_2SO_4$ solubility data of NELSON *et al.*[4], we would expect most of the C' components to be present in substantially reduced amounts, except for C'9. Efforts at further purification of this fraction are in progress.

In our efforts to develop this method, we have made a number of observations that may be useful in the application of solubility chromatography to other problems.

We have compared two types of gel, *viz.*, polyacrylamide (Bio-Gel P-10) and dextran (Sephadex G-50) gels. Although some conditions may indicate the use of polyacrylamide[1], Sephadex G-50 would appear to be the medium of choice for work at high ionic strength and consequent low water activity, which affects the water regain of the gel. On changing from 1 *M* NaCl to 3.5 *M* $(NH_4)_2SO_4$, the bed volume of a Bio-Gel P-10 column dropped by 50%, whereas that of a Sephadex G-50 column decreased by 30%; the latter gel showed little or no volume change below 2.7 *M* $(NH_4)_2SO_4$, the range in which fractionation took place in this study. We have found that the particle size of the gel is of some importance; as in other applications of gel filtration, the sharpest separation is attained with the smallest particle size. No other gels were evaluated.

Since separation is occurring on the basis of precipitation and dissolution, which are relatively slow processes, one might expect the flow rate to play a crucial role. This has turned out to be the case; we found that flow rates above 2.5 cm/h led to extensive tailing, indicating that equilibrium was not being attained. For this reason, the use of a coarse gel particle size provides no advantage over the fine particle size, since the higher flow rate afforded thereby would be detrimental to separation.

In this study, we have examined two methods of applying the protein sample to the column. In addition to the method described, we tried placing the protein on the gel in the form of a slurry of precipitate before applying the gradient, hoping that the gradient would selectively dissolve the precipitate as it passed through; this would have avoided altogether the need to handle the protein in solution. This method of application did not yield satisfactory results because the liquid flowing through the precipitate created channels, leaving behind islands of undissolved protein. The method described is therefore the method of choice.

The slope of the $(NH_4)_2SO_4$ concentration gradient has a predictable effect. In run XXXVI-3 (Table II) a gradient was used which was shallower in the region of C'2 elution than the one described in Table I. In this run the C'2 was eluted at nearly the same position, but in a zone that was approximately twice as broad as in the other runs, with a slight loss in purification efficiency. Steeper gradients were not tried, but stepwise elution appears undesirable—we have found that a 0.5 *M* reduction in $(NH_4)_2SO_4$ concentration in a single step produces marked tailing in both protein and $(NH_4)_2SO_4$ concentrations, extending over more than a liter, in the effluent. This

artefact may be due to a failure to attain equilibrium across a sharp decrease in $(NH_4)_2SO_4$ concentration; however, this argument does not explain the tailing with respect to $(NH_4)_2SO_4$ concentration.

Fig. 1 shows that some proteins have migrated through the gradient and penetrated fairly deeply into the region on the column where the $(NH_4)_2SO_4$ concentration is 2.7 *M*, without emerging with the void volume. This observation can be explained on the assumption that the proteins in question are partially soluble in 2.7 *M* $(NH_4)_2SO_4$; if the column is equilibrated with 3.5 *M* $(NH_4)_2SO_4$ before application of the sample, all the protein emerges in the gradient. In the case of C'2 the concentration of $(NH_4)_2SO_4$ with which the column is equilibrated has a marked effect on migration. In spite of the fact that under the conditions described, the C'2 emerges in the gradient at 2.28 *M* $(NH_4)_2SO_4$, it will elute only slightly behind the void volume of a column equilibrated with 2.5 *M* $(NH_4)_2SO_4$. On a column equilibrated with 2.6 *M* $(NH_4)_2SO_4$, part of the C'2 emerges ahead of the gradient, the rest early in the gradient. In either case, the C'2 zone is very broad, extending over a liter or more of effluent, and purification is poor. These observations may be summarized by suggesting that in a critical range of $(NH_4)_2SO_4$ concentrations, it is possible to achieve a true counter-current process, but that a shallow gradient will produce the best results.

It seems likely that the results described in this report for solubility chromatography in concentrated $(NH_4)_2SO_4$ solutions can be generalized to other precipitants, including organic solvents; the latter could be used in conjunction with the lipophilic Sephadex LH-20. We have explored the applicability of solubility chromatography in ethanol on Sephadex LH-20 as a step in the purification of C'2, but have been discouraged from pursuing it further by the occurrence of artefacts and by very poor recoveries.

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CHROM. 3900

STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

XIV. CHROMATOGRAPHY OF NORMAL AND ABNORMAL HUMAN HEMOGLOBIN TYPES ON CM-SEPHADEX

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SUMMARY

A new chromatographic procedure for the separation and quantitation of human hemoglobin components is described. The method utilizes columns of carboxymethyl–Sephadex G-50 and 0.05 *M* Tris–maleic acid buffers as developers; application of a simple pH gradient is preferred over stepwise elution. The procedure allows the separation of Hb-A_2 from the hemoglobins S and C, and of Hb-D_2 from the slow moving hybrid component SDα. Fetal hemoglobin is eluted in front of the normal Hb-A. The minor hemoglobins of normal red cell hemolysates were fractionated into at least four components, well separated from the major hemoglobin component. The procedure has the disadvantage of requiring several days to complete the chromatographic separations.

INTRODUCTION

The procedures most commonly used for the chromatographic separation of normal human hemoglobin types and of their variants make use of the anion exchangers DEAE-cellulose[1,2] and DEAE-Sephadex[2,3], and of the cation exchangers Amberlite IRC-50[4–7] and CM-cellulose[8–10]. Each of these techniques has their advantages and disadvantages. Chromatography on Amberlite IRC-50, for instance, is particularly useful for the isolation of the various minor hemoglobin components from normal adult and cord blood red cell hemolysates, while DEAE-Sephadex chromatography offers a rapid and reliable technique for the isolation and quantitation of hemoglobin variants from blood samples of patients with different hemoglobinopathies. In this paper we introduce a CM-Sephadex chromatographic procedure, which is of value for the isolation and quantitation of certain major and minor hemoglobin components with closely identical electrophoretic mobilities. The method does not lend itself easily to routine application because of the time required to complete a chromatogram. This disadvantage makes a careful selection of the material to be analyzed a necessity.

MATERIALS AND METHODS

Hemoglobin samples

Blood samples were obtained from the following sources:

(1) Normal adults and cord blood samples from Negro babies.

(2) One cord blood sample from a Negro baby with a heterozygosity for the β chain variants S and C (patient N.C.).

(3) One homozygous sickle cell anemia patient (J.G.) and one patient with SC disease (patient S.C.).

(4) One heterozygous β-thalassemia carrier with elevated levels of Hb-A_2 and Hb-F (patient W.O.).

(5) One hemoglobin C-β-thalassemic individual (patient G.S.); starch gel electrophoretic examination showed the presence of small amounts of Hb-A and Hb-F in addition to Hb-C.

(6) One adult patient with a heterozygosity for an α chain variant (Hb-Dα) and for the β chain variant Hb-C (patient L.R.). Four major hemoglobin fractions were demonstrated by starch gel electrophoresis, namely Hb-A or $\alpha_2\beta_2$, Hb-Dα or $\alpha_2 D\beta_2$, Hb-C or $\alpha_2\beta_2 C$, and Hb-CDα or $\alpha_2 D\beta_2 C$. Two additional minor fractions, Hb-A_2 or $\alpha_2\delta_2$ and Hb-D_2 or $\alpha_2 D\delta_2$, were not observed with this technique because of the identical electrophoretic mobilities of Hb-C and Hb-A_2, and of Hb-CDα and Hb-D_2. The father of this individual (patient R.R.) exhibited a homozygosity for Hb-C and a heterozygosity for Hb-Dα; only two components were observed by starch gel electrophoresis. Blood samples from both patients were kindly supplied by Dr. R. B. SQUIRES, Baptist Hospital, Pensacola, Fla.

(7) One individual with a heterozygosity for Hb-C-Harlem (patient G.S.). This β chain variant has been identified as an abnormality with two amino acid residue substitutions, namely a Glu to Val substitution in position 6 and an Asp to Asn substitution in position 73 (ref. 11). A blood sample from an individual with an apparently similar Hb-C-Harlem heterozygosity, but in combination with a Hb-C ($\alpha_2\beta_2{}^{6}\mathrm{Lys}$) heterozygosity, was also studied. Starch gel electrophoresis of the hemoglobin of this last patient (patient P) showed only one component in the position of Hb-C. The blood samples of patients S and P were made available to us by Dr. W. A. SCHROEDER, California Institute of Technology, Pasadena, Calif.

(8) The hemoglobins F_0, A_0, A_2, S_0 and C_0, isolated by DEAE-Sephadex chromatography[2,3]. The minor hemoglobins A_{Ia+b} and A_{Ic}, isolated by Amberlite IRC-50 chromatography[7], were kindly supplied by Dr. B. F. HORTON, University of Tennessee, Knoxville, Tenn.

CM-Sephadex chromatography

CM-Sephadex (C-50, capacity: 4.5 $\pm$ 0.5 mequiv/g, Pharmacia Fine Chemicals, lot No. 9494) was used. The cation exchanger was equilibrated with the first developer and stored at room temperature. The buffer was changed once weekly. The developers were 0.05 *M* Tris-(hydroxymethyl)aminomethane–maleic acid buffers of pH varying between 6.5 and 8.0. These developers were prepared from 1 *M* stock solutions of both chemicals, by titrating the 0.05 *M* Tris solution with the concentrated maleic acid solution to the desired pH value. Each developer also contained 100 mg KCN/1000 ml. A column of 60 $\times$ 0.9 cm was used in most experiments; the resin column was usually

equilibrated with a pH 6.5 developer, and in certain instances with a pH 6.7 developer. Seventy to 150 mg of hemoglobin (in 2 to 3 ml), dialyzed overnight at 4° against the first developer, was chromatographed. A pH gradient was applied by supplying a buffer of higher pH value (usually 7.0) from a separatory funnel to a 250 ml mixer which contained the first developer. The selection of additional developers and the time of introduction depended on the elution pattern observed. The flow rate was maintained at approx. 20 ml/h; an increase was not possible due to an undesirable packing of the column under increased pressure. The time required to complete the chromatogram (5 to 7 days!) made it necessary to run the analyses at 4°. The effluent was collected in 8 ml fractions and analyzed as described before[1–3,8–10]. Measurements of the pH of the effluent were made at room temperature with a Radiometer PH-4 pH meter within 24 h after collection.

Additional techniques

Several isolated hemoglobin components were further characterized by starch gel electrophoresis and by analyses of their total amino acid composition. The components were concentrated by ultrafiltration under reduced pressure at 4°, dialyzed overnight against distilled water, whereafter the appropriate amounts were hydrolyzed for 24 h with 6 *N* HCl at 110° under reduced pressure. Amino acid analyses were made with a Spinco model 120B automatic amino acid analyzer. Procedures for the preparation of hemolysates, starch gel electrophoresis, analytical and preparative chromatography using DEAE-Sephadex have been described before[2,3,12].

RESULTS AND DISCUSSION

Separation of an artifical mixture of the hemoglobins A, F, S, C and A_2

The chromatogram was initially developed with a 6.7 to 7.0 pH gradient; the pH 7.0 developer was replaced in the separatory funnel by similar buffer solutions but with pH values of 7.3, 7.5, 7.7 and 8.4 after approximately 350, 1100, 1450 and 1600 ml of developer passed through the column, respectively. Fig. 1 illustrates the chromatogram; the five major hemoglobin fractions were eluted at pH values as indicated. The identity of each component was determined by starch gel electrophoresis; the relative electrophoretic mobilities, presented in Fig. 2, indicated a sequence of F, A, A_2, S and C in the elution of these components. The identities of components III (or Hb-A_2) and V (or Hb-C) were confirmed by amino acid analyses of their 24 h acid hydrolysates. The electrophoretic pattern also indicated that each component was pure, except the Hb-A_2 and Hb-S fractions, which were contaminated to a minor extent with each other. The elution pattern is rather different from that observed in CM-cellulose chromatography, namely A, F, S, C + A_2 (ref. 9). Application of CM-Sephadex chromatography, therefore, offers an unique opportunity to separate the hemoglobins A_2 and C from red cell hemolysates. The separation of Hb-F and Hb-A is comparable to that observed in Amberlite IRC-50 chromatography.

Separation of minor hemoglobin components from normal red cell hemolysates

Approximately 130 to 150 mg of hemoglobin was chromatographed. The majority of the hemolysates were freshly prepared, while others were either aged for 40 days at 4° or treated with oxidized glutathione, as described in previous papers of

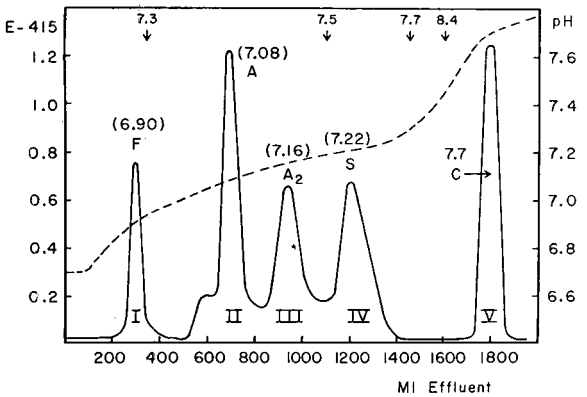

Fig. 1. Chromatography of an artificial mixture of the hemoglobins F, A, A_2, S and C on a column of CM-Sephadex at 4°. An initial pH gradient with 0.05 *M* Tris–maleic acid developers of pH 6.7 to 7.0 was applied; the second buffer of this gradient system was replaced by additional developers with increasing pH values (7.3, 7.5, 7.7, 8.4) as indicated. The values between parentheses represent the pH elution values of the individual zones. The broken line represents the pH of the effluent. The flow rate was 20 ml/h.

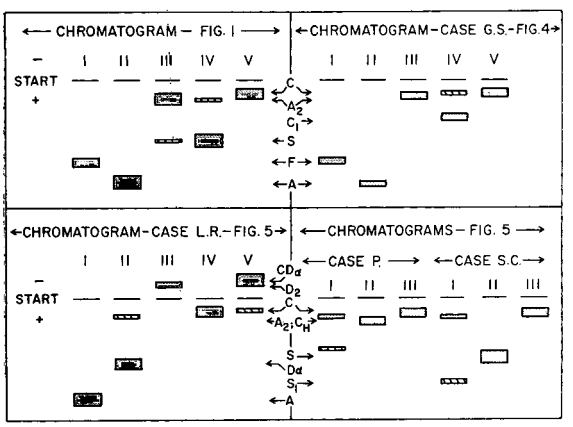

Fig. 2. Schematic presentation of the mobilities of isolated hemoglobin components in starch gel electrophoresis.

this series[13,14]; such treatment will result in the formation of a mixed disulfide, which has been identified as $\alpha_2(SH)_2\beta_2$ $(SH)_2(SSG)_2$ (ref. 13). The chromatograms were developed with a 6.5 to 7.0 pH gradient, while a pH 7.4 buffer was introduced after approximately 1500 ml of developer passed through the columns. Four, and probably five, minor components were observed in a normal freshly prepared hemolysate; these fractions were eluted at pH values of 6.80, 6.85, 6.92 and 7.01, respectively (Fig. 3). The figure also lists the ranges of the relative amounts for each component, calculated from 10 chromatograms. Aging of the hemolysate and incubation with oxidized glutathione resulted in a marked increase in component IV (chromatograms 2

and 3 of Fig. 3), which identified this fraction as the A-GSSG complex. Chromatography of the Hb-A_{Ia+b} and of the Hb-A_{Ic} components, isolated by Amberlite IRC-50 chromatography, identified the components II and III (chromatograms 4 and 4a of Fig. 3). The nature of the minute component I and of component IIIa is not known; amino acid analyses of 24 h hydrolysates of component IIIa indicated the probable presence of a fetal hemoglobin component. A comparison of these data with those observed in other types of cation exchange chromatography indicates a notable improvement of the resolution of the minor hemoglobin fractions over that seen in CM-cellulose chromatography[9,10]. The elution pattern is more or less comparable to that observed in Amberlite-IRC-50 chromatography.

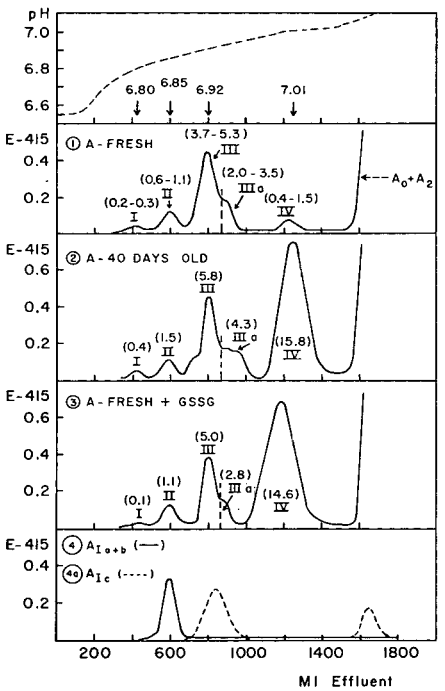

Fig. 3. Chromatography of the minor hemoglobin components on columns of CM-Sephadex. (1) Freshly prepared hemolysate. (2) Same hemolysate after storage at 4° for 40 days. (3) Same hemolysate after treatment with oxidized glutathion. (4) Hb-A_{Ia+b} and (4a) Hb-A_{Ic}, isolated by Amberlite IRC-50 chromatography. The values between parentheses represent the relative amounts of the individual zones; the elution pH value of each individual zone is given at the top of the first chromatogram. For further details see text.

The separation of fetal hemoglobin components from cord blood hemolysates, and from red cell hemolysates of patients with SS anemia, β-thalassemia trait, and β-thalassemia-Hb-C disease

These chromatograms were initially developed with a pH 6.5 to 7.0 gradient, while the pH 7.0 buffer was replaced by similar buffer solutions but with pH values of 7.3, 7.5 and 8.0 after approximately 1500, 2300 and 2600 ml passed through the column, respectively. Each sample was simultaneously analyzed by DEAE-Sephadex

chromatography[3]. The chromatograms obtained with these two techniques are compared in Fig. 4; case N.C. represents a newborn baby with a heterozygosity for both Hb-S and Hb-C, case J.G. a patient with sickle cell anemia, case W.O. a patient with β-thalassemia trait, and case G.S. a patient with Hb-C-β-thalassemia disease. Careful examination of these chromatograms allows the following conclusions: (a) Fetal hemoglobin was observed as two, incompletely separated, components, which are eluted at pH values of 6.91 and 6.94 respectively. The total amounts of fetal hemoglobin calculated from the CM-Sephadex chromatograms, corresponded rather well with those calculated from the DEAE-Sephadex chromatograms. (b) The separation of the Hb-A_2 from Hb-S and Hb-C was (almost) complete, thus allowing its quantitation in patients with a Hb-C heterozygosity or homozygosity. (c) The resolution observed by CM-Sephadex chromatography was in most instances greater than that seen in the DEAE-Sephadex chromatograms. This is particularly apparent in the chromatograms of the β-thalassemic individual (case W.O.) and of the Hb-C-β-thalassemic patient (case G.S.). The electrophoretic mobilities of the components I through V of the chromatogram from case G.S. are presented in Fig. 2; the observations made are in agreement with those described for the isolated fractions of the

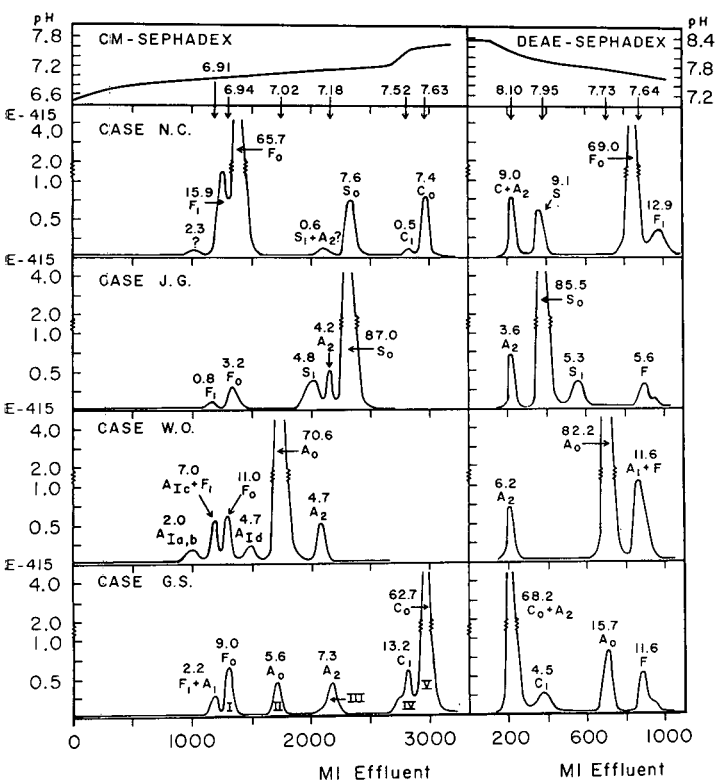

Fig. 4. Chromatography of red cell hemolysates containing fetal hemoglobin on columns of CM-Sephadex and of DEAE-Sephadex. Case N.C.: cord blood from a Negro baby with an heterozygosity for the hemoglobins S and C. Case J.G.: sickle cell anemia. Case W.O.: β-thalassemia trait. Case G.S.: Hb-C-β-thalassemia trait. For further details see legend to Fig. 3 and text.

chromatogram given in Fig. 1. (d) The correlation between the relative amounts of the various hemoglobin components, calculated from the CM-Sephadex and the DEAE-Sephadex chromatograms was rather good, except for that of Hb-A_0 in case G.S., which remains unexplained.

Chromatographic separation of some rare hemoglobin components

The cases, of which chromatograms are presented in Fig. 5, concerned case L.R. with a heterozygosity for the β chain variant Hb-C and for the α chain variant Hb-D; case R.R. with a homozygosity for Hb-C and a heterozygosity for Hb-Dα; case G.S. with a heterozygosity for Hb-C-Harlem; case P with a heterozygosity for Hb-C-Harlem and for Hb-C, and case S.C. with Hb-S-Hb-C disease. The chromatograms were developed in a similar way as described for those presented in Fig. 4. The following remarks seem appropriate:

Case L.R. The separation of the hemoglobins A,Dα, C_0 and the hybrid compo-

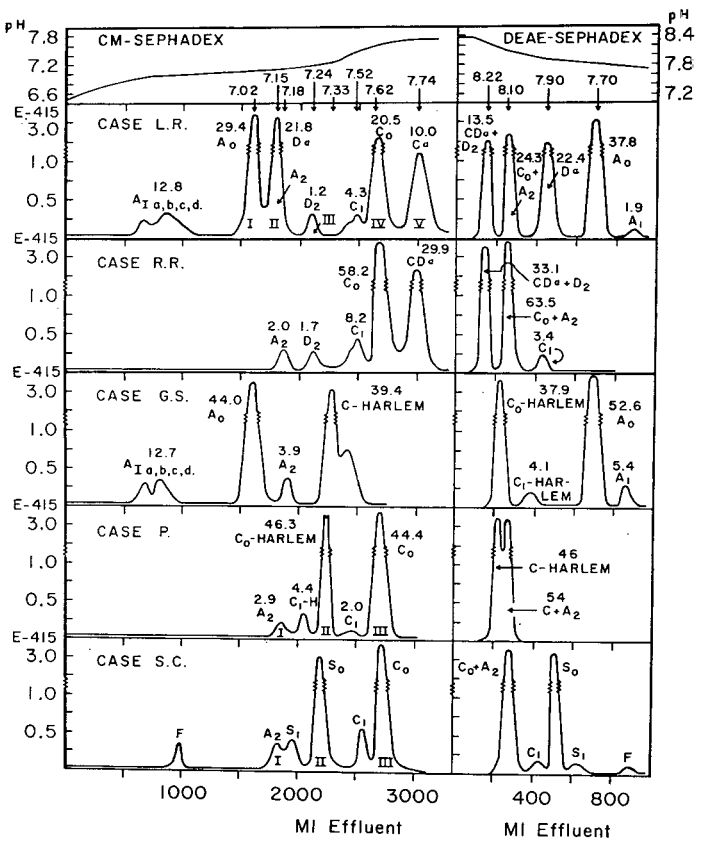

Fig. 5. Chromatography of red cell hemolysates containing some rare hemoglobin types on columns of CM-Sephadex and of DEAE-Sephadex. Case L.R.: heterozygosity for the α chain variant Dα and the β variant Hb-C. Case R.R.: heterozygosity for the α chain variant Dα and homozygosity for the β chain variant Hb-C. Case G.S.: heterozygous C-Harlem carrier. Case P.: Hb-C-Hb-C-Harlem heterozygosity. Case S.C.: Hb-S-Hb-C disease. For further details see legend to Fig. 3. and text.

nent CDα was complete in both types of chromatography. CM-Sephadex chromatography allowed also the isolation of Hb-D_2 (or $\alpha_2 D\delta_2$) but not of Hb-A_2, which had a mobility similar to that of Hb-Dα (or $\alpha_2 D\beta_2$). The identity of several of these isolated fractions is based on the differences in their electrophoretic mobilities (Fig. 2); the presence of Hb-A_2 in the isolated component II is clearly demonstrated by this procedure.

Case R.R. The absence of normal Hb-A and of Hb-Dα was confirmed in both chromatograms. CM-Sephadex chromatography allowed the complete separation of the minor components Hb-A_2 and Hb-D_2; the relative amounts of these hemoglobins were 2.0 and 1.7 % respectively. Hb-D_2 was eluted at approximately the same pH value as observed for Hb-S (Fig. 1).

Case G.S. The elution pH value for Hb-C-Harlem was found to be approximately 7.33, thus allowing a complete separation of this variant and of the minor Hb-A_2 component.

Case P. A rather incomplete separation of Hb-C-Harlem and Hb-C was observed by DEAE-Sephadex chromatography; the separation of these two components and of Hb-A_2 was complete in the CM-Sephadex chromatogram. The level of Hb-A_2 in this blood sample was 2.9 %; no trace of Hb-F was observed. The electrophoretic mobilities of the isolated components I, II and III are shown in Fig. 2.

Case S.C. This chromatogram closely resembled that of patient P; it may be that Hb-S was eluted slightly faster than Hb-C-Harlem. The separation of Hb-A_2 and the minor Hb-S component was incomplete, preventing an acceptable calculation of the relative amount of Hb-A_2. The relative mobilities of the fractions I, II and III in starch gel electrophoresis are also presented in Fig. 2. It is noteworthy that a small amount of Hb-F (1.6 %) was present in this blood sample.

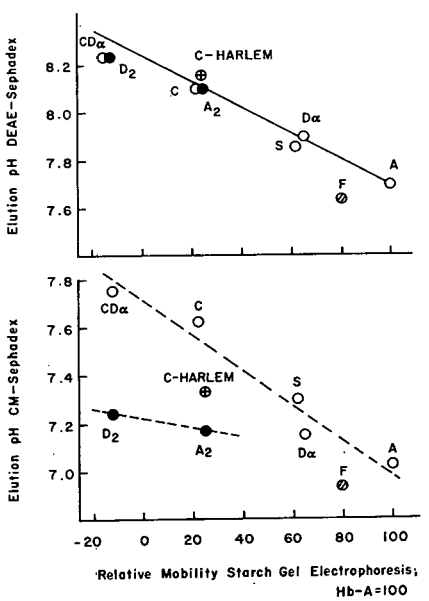

Fig. 6. The relationship between the relative mobilities of various hemoglobin variants and their elution pH values in CM-Sephadex and in DEAE-Sephadex chromatography.

In reviewing the several chromatograms it becomes evident that CM-Sephadex chromatography shows a selectivity, which is considerably greater than that observed by the DEAE-Sephadex chromatographic procedure. This is even more apparent when the relative mobilities of the various components in starch gel electrophoresis are plotted against their respective elution pH values in both types of chromatography (Fig. 6). The separation of the hemoglobin components (with the exception of Hb-F) by DEAE-Sephadex chromatography seems primarily to be based on charge differences between the molecules; additional properties of the structurally different hemoglobin components apparently influence the adsorption of the proteins to the CM-Sephadex cation exchanger to a much greater extent than is the case for the DEAE-Sephadex anion exchanger. This phenomenon can successfully be used for a complete separation of components showing (almost) identical mobilities in starch gel electrophoretic separations, such as Hb-CDα and Hb-D_2, Hb-C and Hb-C-Harlem and Hb-A_2, and perhaps Hb-S and Hb-D.

ACKNOWLEDGEMENT

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CHROM. 3896

ENTSALZUNG VON STREPTOMYCIN AN SEPHADEX G-10

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SUMMARY

Desalting of streptomycin on Sephadex G-10

The behaviour of mixtures consisting of streptomycin-sulphate and alkali metal salts in gel filtration with Sephadex G-10 was investigated. Elution with deionized water results in a practically saltfree product. Yield is about 95 %. Streptomycin is eluted in two fractions with deionized water. The normal molecular-sieve effect is obtained using buffer-solutions for elution.

EINLEITUNG

Seit geraumer Zeit wird zur Auftrennung von Substanzgemischen, deren Bestandteile sich in ihrer Molekülgrösse unterscheiden, neben Dialyseverfahren die Methode der Gelfiltration angewendet[1]. Zur Abtrennung oder Fraktionierung niedermolekularer Verbindungen, wozu auch die Entsalzung organischer Substanzen zu rechnen ist, sind Gele mit hohem Vernetzungsgrad geeignet[2–9]. Namentlich dicht vernetzte Gele zeigen aber neben dem üblichen Molekülsiebeffekt Adsorptionserscheinungen, die auf Wechselwirkungen der gelösten Stoffe mit der Gelmatrix zurückzuführen sind.

GELOTTE[10] beobachtete eine Adsorption heterozyklischer und aromatischer Verbindungen an Sephadex G-25. In Abhängigkeit von der Ionenstärke und dem pH-Wert des Eluens fand er eine Retardierung bei einigen starken Basen und einen teilweisen Ausschluss saurer niedermolekularer Verbindungen vom Gelinneren.

LINDQVIST[11] berichtete über die Auftrennung der Komponenten von Puffersalzen während der Gelfiltration mit Sephadex G-25. Für einige der beobachteten Nebeneffekte werden kleine Mengen an Carboxylgruppen verantwortlich gemacht, die in der Gelmatrix enthalten sind. Diese Dextrangele erhalten dadurch Eigenschaften schwach saurer Kationenaustauscher mit geringer Austauschkapazität[12,13]. Mitunter wird durch einen solchen Nebeneffekt die gewünschte Auftrennung von Substanzgemischen erreicht[14,15]. Im Verlaufe der Herstellung von [^{15}N]-Streptomycin mussten einige 100 mg des Rohproduktes von der zwei–dreifachen Menge Alkalisalzen

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befreit werden. Im Hinblick auf die genannten Anomalien, die bei dicht vernetzten Dextrangelen auftreten können, wurde das Verhalten von definierten Gemischen aus Streptomycinsulfat (Sm-Sulfat) und Alkalisalzen bei der Entsalzung mit Sephadex G-10 näher untersucht.

MATERIAL UND METHODEN

Trennsäule

Sephadex G-10 (Pharmacia AB, Uppsala) wurde über Nacht in deionisiertem Wasser gequollen und in eine Säule von 1.44 cm Durchmesser eingefüllt. Die Füllhöhe des Gelbettes betrug nach 48-stündigem Durchlaufen von deionisiertem Wasser 113 cm. Alle Trennungen wurden mit dieser Säule durchgeführt.

Säulenvolumina

Das Gesamtvolumen V_t des Gelbettes betrug 184 ml. Das äussere Volumen V_o wurde mit Stärke als Testsubstanz zu 73 ml bestimmt. Das innere Volumen V_i (68 ml) wurde berechnet* nach

$$V_i = \frac{d \cdot W_r}{W_r + 1} (V_t - V_0).$$

Zur Ermittlung des Elutionsvolumens V_e wurden die Peak-Maxima der Elutionskurven verwendet.

Testgemische

10–200 mg Sm-Sulfat (VEB Jenapharm, Jena) wurden mit 10–200 mg NaCl, Na_2SO_4, KCl oder K_2SO_4 versetzt und in 1 ml des verwendeten Elutionsmittels gelöst.

Elutionsbedingungen

Das Probevolumen betrug in allen Versuchen 1 ml. Die Elution erfolgte mit deionisiertem Wasser oder 0.2 *M* Ammoniumacetat-Puffer (pH 6.5) bei Zimmertemperatur. Die Fliessgeschwindigkeit wurde mit einer Mariotte'schen Flasche oder einer Feindosierpumpe auf 8–12 ml/h eingestellt und das Eluat in 1–2 ml-Fraktionen gesammelt. Die Leitfähigkeit des Eluates wurde kontinuierlich verfolgt.

Analysenmethoden

Qualitative Nachweise erfolgten mit aliquoten Teilen der Fraktionen.
Streptomycin: Sakaguchi-Reaktion
Alkalimetall-Ionen: Flammenfärbung (Handspektroskop)
Chlorid-Ionen: als AgCl
Sulfat-Ionen: als $BaSO_4$.

Die quantitative Bestimmung des Streptomycins wurde nach MONASTERO[16] durchgeführt, Chlorid volumetrisch nach MOHR mit 0.01 *N* oder 0.1 *N* $AgNO_3$ bestimmt. Sulfat, Natrium und Kalium wurden nur qualitativ nachgewiesen.

* Die Werte für d (Dichte der gequollenen Gelpartikel) und W_r (Wasseraufnahmevermögen) wurden der Pharmacia Literatur entnommen.

ERGEBNISSE

Elution mit deionisiertem Wasser

Mischungen aus Sm-Sulfat und Natriumsulfat oder Kaliumsulfat werden an Sephadex G-10 mit deionisiertem Wasser als Elutionsmittel getrennt (Fig. 1). Natriumsulfat folgt unmittelbar auf Sm-Sulfat. Wie aus dem Leitfähigkeitsprofil hervorgeht, sind die letzten Sm-Fraktionen nicht salzfrei. Vor dem Leitfähigkeitsanstieg vereinigte Streptomycin-Fraktionen (etwa 95 % der eingesetzten Menge) ergaben einen Glührückstand von weniger als 1 mg. Die letzten Salzspuren werden mit deionisiertem Wasser nur schleppend eluiert.

Ist der Salzbestandteil in Form von Chloriden enthalten, erhält man den in Fig. 2 dargestellten Elutionsverlauf. Es treten zwei Streptomycin-Peaks auf. Ein Teil

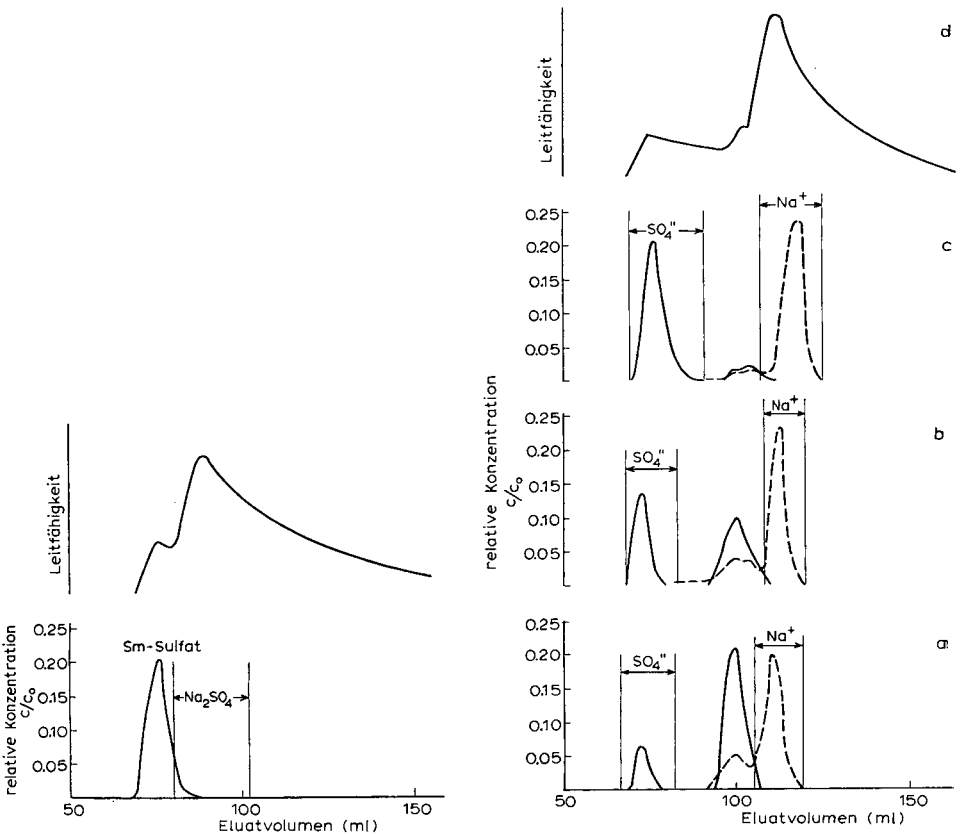

Fig. 1. Elutionsverlauf bei der Entsalzung von 200 mg Sm-Sulfat an Sephadex G-10. Salzgehalt: 100 mg Na_2SO_4. Elution mit deionisiertem Wasser bei Zimmertemperatur. Fliessgeschwindigkeit 10 ml/h. Dargestellt ist das Verhältnis der in 1–2 ml-Fraktionen enthaltenen Menge c zur eingesetzten Menge c_0 (200 mg Sm-Sulfat). Die Leitfähigkeit wurde kontinuierlich gemessen.

Fig. 2. Entsalzung von Gemischen aus Sm-Sulfat und NaCl an Sephadex G-10. Elutionsmittel: deionisiertes Wasser. (a) = 10 mg Sm-Sulfat + 10 mg NaCl; (b) = 20 mg Sm-Sulfat + 20 mg NaCl; (c) = 200 mg Sm-Sulfat + 100 mg NaCl; (d) Leitfähigkeit. (———) = Relative Sm-Konzentration; (— — — —) = relative Chlorid-Konzentration.

des Antibiotikums wird als Chlorid eluiert. Der Mengenanteil in beiden Fraktionen ist abhängig von den eingesetzten Streptomycin-Mengen, jedoch im untersuchten Konzentrationsbereich weitgehend unabhängig vom Molverhältnis der Anionen im Gemisch (Tabelle I). Das Molverhältnis Chlorid: Streptomycin in der zweiten Sm-Fraktion ist bei maximaler Sm-Konzentration wenig grösser als 3, dem theoretischen Wert für Sm-Chlorid. Natriumchlorid wird zuletzt eluiert. Auch hier wird der Leitfähigkeitswert des verwendeten deionisierten Wassers nur langsam erreicht.

Kaliumsalze verhalten sich bei der Entsalzung von Sm-Sulfat an Sephadex G-10 analog den entsprechenden Natriumsalzen. Besonders deutlich ist die Anionenselektivität des Gels (Fig. 3). Die Elutionsvolumina sind bei verschiedenen Ausgangskonzentrationen und über längere Zeitabstände gut reproduzierbar (Tabelle II).

TABELLE I

MENGENVERHÄLTNIS Sm-SULFAT: Sm-CHLORID IM ELUAT BEI VERSCHIEDENEN AUSGANGSMENGEN

Eingesetztes Sm-Sulfat (mg)	*Molverhältnis Chlorid: Sulfat im Gemisch*	*Mengenverhältnis Sm-Sulfat: Sm-Chlorid im Eluat*
10	8:1	1:5
20	8:1	1:1
200	4:1	9:1

TABELLE II

ELUTIONSVOLUMINA V_e BEI ELUTION MIT DEIONISIERTEM WASSER

	V_e (ml)	$K_d = \frac{V_e - V_0}{V_i}$
Sm-Sulfat	73 ± 3	0
Na_2SO_4	93	0.294
Sm-Chlorid	103 ± 3	0.441
NaCl	115 ± 4	0.617

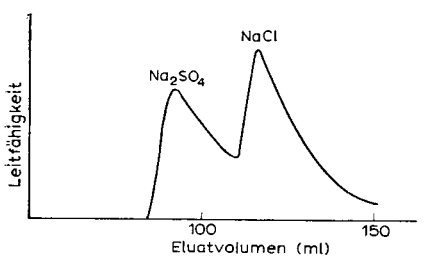

Fig. 3. Auftrennung von Sulfat und Chlorid an Sephadex G-10. Aufgegebene Menge: 20 mg Na_2SO_4 + 20 mg NaCl. Elution mit deionisiertem Wasser. Fliessgeschwindigkeit 8 ml/h.

Elution mit Pufferlösung

Die eine normale Molekülsiebwirkung des Gels überlagernden Effekte können häufig durch Verwendung von Elektrolytlösungen als Elutionsmittel vermieden werden[10]. Zur Entsalzung ist der Einsatz flüchtiger Puffer erforderlich. Die Elution wurde mit 0.2 *M* Ammoniumacetat-Puffer von pH 6.5 durchgeführt. Der Elutionsverlauf ist in Fig. 4 dargestellt. Befindet sich die Trennsäule im Wasser-Gleichgewicht, treten zwei Streptomycin-Peaks auf. Der erste ist Sm-Sulfat, er eilt der Puffer-Front voraus. Im Gleichgewicht mit der Pufferlösung ist zu erwarten, dass Kationen als Acetate, Anionen als Ammoniumsalze in der Lösung vorliegen. Dementsprechend erfolgt eine Veränderung der Elutionsvolumina gegenüber den bei deionisiertem Wasser beobachteten Werten (Tabelle III).

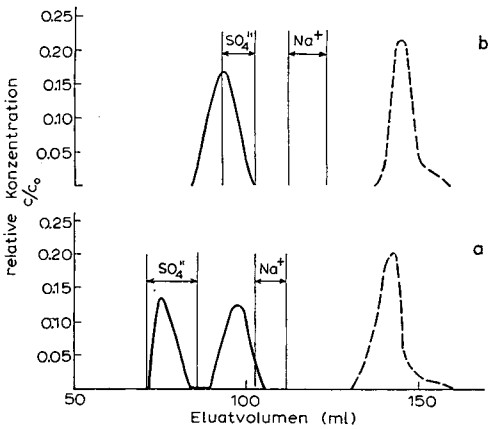

Fig. 4. Entsalzung von Sm-Sulfat an Sephadex G-10. Aufgegeben: 20 mg Streptomycinsulfat + 20 mg NaCl. Elution mit 0.2 *M* Ammoniumacetat-Puffer, pH 6.5. (———) = Relative Sm-Konzentration; (— — — —) = relative Chlorid-Konzentration. (a) = Säule im Wasser-Gleichgewicht; (b) Säule im Puffer-Gleichgewicht.

TABELLE III

ELUTIONSVOLUMINA V_e BEI ELUTION MIT 0.2 *M* AMMONIUMACETATPUFFER

	V_e (*ml*)	$K_d = \frac{V_e - V_0}{V_i}$
Streptomycin	92 ± 4	0.279
$(NH_4)_2SO_4$	97 ± 3	0.353
Na-acetat	115	0.624
NH_4Cl	140 ± 7	0.985

DISKUSSION

Die Elutionsfolge der untersuchten Substanzen entspricht sowohl in den Versuchen mit deionisiertem Wasser als auch mit Pufferlösung der zu erwartenden, wenn man die Grösse der hydratisierten Kationen und Anionen als bestimmend für den beobachteten Trenneffekt ansieht. Jedoch lässt sich die Position des zweiten Sm-Peaks bei Elution mit deionisiertem Wasser damit nicht erklären. Geringe Mengen an Chlorid sind bereits zwischen beiden Streptomycin-Fraktionen nachweisbar. Leitfähigkeitsprofil und relative Chloridionenkonzentration im Eluat deuten darauf hin, dass dieser Teil des Streptomycins von der Gelmatrix retardiert und vom nachfolgenden Salz eluiert bzw. verdrängt wird. Offenbar ist für die Auftrennung des Streptomycins in zwei Fraktionen weniger eine Molekülsieb-Wirkung verantwortlich als vielmehr das Vorhandensein von zum Kationenaustausch befähigten Gruppen in der Gelmatrix[10,13]. Dieser Effekt kann nur dann deutlich werden, wenn sich die Elutionsvolumina von Streptomycin und dem als Elutionsmittel wirkenden Salz wesentlich unterscheiden, wie im Falle von NaCl. Da keine Beziehung besteht zwischen dem Mengenanteil in beiden Sm-Fraktionen und dem Molverhältnis der Anionen im Gemisch, kann es sich nicht um die Auftrennung der im Gleichgewicht gemäss

$$Sm_2(SO_4)_3 + 6\ NaCl \rightleftharpoons 3\ Na_2SO_4 + 2\ SmCl_3$$

vorliegenden Komponenten handeln. Zudem würde die Molekülsiebwirkung des Gels immer die Bindung des grössten hydratisierten Anions an das grösste hydratisierte Kation begünstigen.

Auch das schleppende Absinken der Leitfähigkeit auf den Wert für deionisiertes Wasser spricht für Adsorptionserscheinungen, die sich bei Salzen mit Ionenaustausch erklären lassen. Acetatpuffer als Elutionsmittel normalisieren das Elutionsverhalten. Da hierbei Ammonium- und Acetationen im Vergleich zu den zu trennenden Ionen in grossem Überschuss vorhanden sind, werden die Substanzgemische in Form ihrer Ammoniumsalze (Anionen) bzw. Acetate (Kationen) aufgetrennt. Sm-Acetat und $(NH_4)_2SO_4$ haben vergleichbare Elutionsvolumina, so dass der Streptomycin-Peak teilweise von letzterem überdeckt wird.

Die Selektivität des Gels für Anionen unterschiedlicher Grösse und der im Vergleich dazu geringe Trenneffekt bei verschiedenen Alkaliionen wurde bereits von anderen Autoren beschrieben[17]. Der durch Gelfiltration an Sephadex G-10 erreichbare Grad der Entsalzung von mehr als 99 % bei einer Ausbeute an salzfreiem Streptomycin von etwa 95 % ist für präparative Zwecke ausreichend.

ZUSAMMENFASSUNG

Das Verhalten von Mischungen aus Streptomycin-Sulfat mit Alkalisalzen bei der Gelfiltration an Sephadex G-10 wurde untersucht. Elution mit deionisiertem Wasser ergibt ein praktisch salzfreies Produkt bei einer Ausbeute von etwa 95 %. Streptomycin wird durch kationenaustauschfähige Gruppen in der Gelmatrix mit deionisiertem Wasser in zwei Fraktionen eluiert. Normale Molekülsieb-Wirkung des Gels tritt auf bei Verwendung von Pufferlösungen als Elutionsmittel.

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CHROM. 3886

A SPOT TEST DIAGNOSTIC OF HYDROXYL GROUPS

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SUMMARY

A spot test specifically diagnostic of hydroxyl groups was devised. It makes use of the reagent 4-(*p*-nitrobenzyl)-pyridine, which, on cellulose thin-layer plates, is sensitive to alcohols in the microgram range. Amino, ester, and ether groups do not interfere, but carboxylate and phenolic groups do.

INTRODUCTION

The detection of aziridines[1], alkyl halides[1,2], and methanesulfonates[3] by the use of the reagent 4-(*p*-nitrobenzyl)-pyridine (PNBP) has been a standard procedure for several years. However, the detection of alcohols on thin-layer or paper chromatograms is difficult and generally requires derivatization before chromatography with visualization of the spot under ultraviolet light after treatment with rhodamine B[4,5]. SAWICKI *et al.*[2] reported a spectrophotometric method of assaying for alkyl tosylates and suggested that PNBP might be used for the analysis of alcohols. No other investigators have suggested or used PNBP to detect alcohols.

In the preliminary investigation reported here[6], we attempted to develop a spot test specifically diagnostic of hydroxyl groups and investigated the use of PNBP. We felt that the test had to be sensitive at microgram levels and should be easily and inexpensively performed. Also, the test should be free of interference from other functional groups. Most of these requirements were met.

EXPERIMENTAL

Test chemicals

Most compounds used in the analyses were purchased from commercial sources and were of acceptable purity; any substances below standard purity were purified by known procedures. The criteria for determining purity were melting point, boiling point, index of refraction, and, when necessary, spectroscopic procedures.

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Tosylates

The *p*-toluenesulfonate esters (tosylates) of alcohols were prepared by reaction of the alcohols with the *p*-toluenesulfonyl chloride in pyridine. Procedures for isolating and purifying these derivatives are described in the literature. The physical constants were in agreement with the values cited in the literature.

Chromatography

Thin-layer chromatographic (TLC) plates (5 × 20 cm) were prepared with 0.25-mm thick cellulose (Brinkman, MN-Cellulose powder 300 HR)*. Whatman No. 1 filter paper was used for spot tests on paper. The TLC plates were developed with hexane–ethyl acetate (4:1, v/v) by the ascending technique to a distance of 10 cm.

Reagents

Reagent A: 5 % solution of *p*-toluenesulfonyl chloride in a 1:1 (v/v) mixture of anhydrous pyridine–toluene.

Reagent B: 2 % solution of 4-(*p*-nitrobenzyl)-pyridine (PNBP) in acetone.

Reagent C: aqueous 1 *M* sodium carbonate.

Procedure

The alcohols or alcohol derivatives were spotted on the chromatographic supports in acetone or water solutions with a microliter syringe.

Method A

Solutions of the alcohols were spotted on paper strips or TLC plates that were divided into a grid pattern. The spotting solvent was evaporated with a slow stream of nitrogen, and the plates were transferred to the hood. The test sample was sprayed with reagent A, allowed to stand until nearly dry, and then sprayed with reagent B. After 1 min, the plates were heated with a heat gun for 1 min and then sprayed with a gentle, fine mist of reagent C. The development of a deep blue or purple spot indicated a positive result.

Method B

Acetone solutions (50 μl) containing 50, 20 and 10 μg of the alcohol were transferred to 250-μl test tubes with a microsyringe, and 50 μl of a 15 % solution of *p*-toluenesulfonyl chloride in anhydrous pyridine was added. After 1.5 h at 0°, aliquots of the mixture were spotted on TLC plates and developed in the solvent system described. When the mixture had developed to 10 cm, the plates were allowed to air dry, treated with a spray of reagent B, and heated to 110° for 20 min in an oven or heated with a heat gun for 1–2 min. Subsequent spray treatment of the plates with reagent C caused the development of the positive color.

RESULTS AND DISCUSSION

The mechanism of reaction of PNBP with alkyl tosylates and the resulting reaction products were previously described[2]. The mechanism requires the displace-

* Mention of a proprietary product does not necessarily imply endorsement of this product by the United States Department of Agriculture.

ment of the tosylate by the nucleophile (PNBP) to form an alkyl quaternary pyridinium salt. The product, in alkaline solution, gives a chromogenic substance that is deep blue to royal purple. In the spot test described here, the tosylate was formed *in situ* by allowing the alcohol to react with a toluene and pyridine solution of *p*-toluenesulfonyl chloride that was applied by spraying (reagent A). It was then treated with a solution of PNBP (reagent B) and then with a solution of sodium carbonate (reagent C). The result was the chromogen described.

Modifications such as the substitution of several organic bases for the sodium carbonate and changes in reaction temperature, reaction time, and reaction solvents were attempted, but only the reported conditions and reagents gave the optimum reliability and sensitivity. Several variables involving the structural nature of the alcohols and the response to the reagents were investigated and will be discussed. With more volatile alcohols, care had to be taken to avoid loss of the test alcohol by codistillation: when such substances were assayed, the alcohol spot had to be treated quickly with a gentle spray of *p*-toluenesulfonyl chloride in toluene (reagent A). Appropriate blanks gave negative color reactions.

Chromatographic supports

The common thin-layer supports were investigated, but only cellulose and paper provided a positive color. All reported tests were therefore performed on cellulose TLC plates or paper.

Monofunctional alcohols

Initially, a series of monofunctional alcohols were spotted neat and treated by Method A, and solid alcohols were applied as 10 % solutions of acetone. The reaction was always positive, as shown in Table I. Little difference was apparent in the reactivity or primary, secondary, and tertiary alcohols to PNBP at the levels of concentration used in these preliminary tests. However, a more definitive difference will probably be seen when the rates of displacement are studied.

Selected alcohols were chosen to determine the minimum quantity detectable by spotting graded concentrations of solutions in acetone. Table II shows these results. Also, the tosylates generated *in situ* (Method A) on the chromatographic supports were compared with tosylates purified before spotting (Table III) for sensitivity to the PNBP reagent. Thus, a comparison of the data in Table II with that in Table III

TABLE I

MONOFUNCTIONAL ALCOHOLS SPOTTED NEAT, REACTING WITH THE REAGENTS ACCORDING TO METHOD A

Compound	*Compound*
Cyclopentanol	Cyclopropylmethyl carbinol
1-Methylcyclopentanol	(−)-Isopulegol
1-Ethylcyclopentanol	Decahydro-2-naphthol
2-Methylcyclopentanol	1-Hexen-3-ol
Cyclohexanol	Linalool (3,7-dimethyl-1,6-octadien-3-ol)
cis-4-Methylcyclohexanol	Geraniol (*trans*-3,7-dimethyl-2,6-octadien-1-ol)
trans-4-Methylcyclohexanol	Octanol
Cycloheptanol	Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol)

TABLE II

MINIMUM LIMITS OF DETECTABILITY OF MONOFUNCTIONAL ALCOHOLS SPOTTED ON CELLULOSE THIN-LAYER PLATES (METHOD A)

Alcohol	*Minimum quantity detected* (μg)
Octanol	0.5
Farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol)	5.0
1-Tetradecanol	5.0
1-Hexadecanol	1.0
Phytol	5.0
Cholesterol	10.0
Ergosterol	1.0
Androst-5-ene-3β,17β-diol	Neg. (to 50 μg)

TABLE III

TOSYLATES GIVING POSITIVE RESULTS WITH PNBP

Alcohol tosylate	*Minimum quantity detected* (μg)
1-Hexadecanol	0.1
Farnesol	0.2
1-Tetradecanol	1.0
Octanol	0.2
Phytol	2.0
Cholesterol	0.1
Ergosterol	0.2
Androst-5-ene-3β,17β-diol	0.2

TABLE IV

R_F VALUES OF ALCOHOLS REACTED WITH *p*-TsOCl AND SPOTTED ON CELLULOSE THIN-LAYER PLATES (METHOD B)

Minimum sensitivity: 0.75 μg.

Compound	R_F *values*
1-Methylcyclohexanol	0.46
cis-4-Methylcyclohexanol	0.46
trans-4-Methylcyclohexanol	0.46
Cyclohexanol	0.52
Cycloheptanol	0.52
(−)-Isopulegol	0.52
Borneol	0.60
1-Ethylcyclopentanol	0.62
Geraniol	0.62
D,L-Isoborneol	0.62
1-Cyclopropylethanol	0.64
1-Hexen-3-ol	0.64
(+)-Linalool	0.64
Decahydro-2-naphthol	0.66
1-Methylcyclopentanol	0.70
Cyclopentanol	0.72

indicates that purification of the tosylate increases the sensitivity of the test by a factor of 10. These data also suggest that the source of variation is incomplete tosylation of the alcohol *in situ* and/or partial codistillation of the volatile alcohols with the solvent.

Method B was developed to assure completeness of reaction when very low concentrations of alcohol were assayed. The reaction mixture resulting from Method B was spotted on TLC plates and developed to 10 cm in hexane–ethyl acetate (4:1, v/v). However, chromatography was necessary to separate the ester from the remaining reaction products since these substances interfered with the assay. The method was always sensitive to 0.75 μg of alcohol. The alcohols and the R_F values are listed in Table IV.

Polyhydroxy compounds

The reaction of six polyols (Table V) with the reagents was conducted, and acceptable results were obtained in all tests. We feel that the reaction of 4-(*p*-nitrobenzyl)-pyridine with polyol tosylates would present an interesting study and should be investigated further.

TABLE V

DETECTION AND SENSITIVITY OF POLYHYDROXY COMPOUNDS TO PNBP (METHOD A)

Compound	*Minimum quantity detected* (μg)
D-Glucose	5.0
D-Galactose	5.0
D-Xylose	5.0
D-Sorbitol	5.0
Glycerol	1.0
1,2-Ethanediol	1.0

Reaction in the presence of other functional groups

The influence of other functional groups on the reactivity of the hydroxyl group to the reagent was studied by using a series of amino alcohols, amino acids, phenolic compounds, hydroxylated carboxylic acids, ethers, and esters (Table VI). Tentative conclusions were drawn from the results:

The presence of an amino group does not interfere with the test since a series of five amino alcohols (1–6, Table VI) yielded a positive assay. Reaction of *p*-toluenesulfonyl chloride with amino alcohols yields the bis-derivative (tosylamide and tosylate), but only the tosylate groups are displaced by PNBP[6], a conclusion that is also supported by the negative results observed with phenethylamine and 3,4-dimethoxyphenethylamine (9–10, Table VI), both of which lack a hydroxyl group but carry an amino group that would form a tosylamide.

Phenolic compounds (11, 12, Table VI) gave negative results. In addition, the phenolic group appears to interfere with the assay of an aliphatic hydroxyl group in the side chain since noradrenaline (7, Table VI) did not give the typical blue of the alcohols. In fact, dopamine (8, Table VI), which lacks an aliphatic hydroxyl group, gave similar atypical results.

TABLE VI

INFLUENCE OF OTHER FUNCTIONAL GROUPS ON THE PNBP TEST FOR HYDROXYL GROUPS

No.	*Compound*	*Minimum quantity detected* (μg)	*Color*
1	2-Aminoethanol	positive (1.0)	blue
2	2-(Methylamino)-ethanol	positive (0.75)	blue
3	2-Amino-1-propanol HCl	positive (0.75)	blue
4	2-Amino-1-butanol	positive (0.75)	blue
5	3-Amino-1,2-propanediol	positive (0.75)	blue
6	N,N-Bis(β-hydroxyethyl)-*p*-anisidine	positive (0.5)	blue
7	Noradrenaline HCl [(α-(aminomethyl)-3,4-dihydroxy-benzyl alcohol HCl]	positive (5.0)	grey-brown
8	Dopamine HCl [4-(2-aminoethylpyrocatechol) HCl]	positive (5.0)	grey-brown
9	3,4-Dimethoxyphenethylamine	negative	
10	Phenethylamine	negative	
11	Resorcinol	negative	
12	2-Naphthol	negative	
13	Piperonyl alcohol	negative	
14	4-Aminobutyric acid	negative	
15	Glutamic acid	negative	
16	Proline	negative	
17	Serine	negative	
18	Malonic acid	negative	
19	Succinic acid	negative	
20	Tartaric acid	negative	
21	Citric acid	negative	
22	Phenylacetic acid	negative	
23	Benzoic acid	negative	
24	Diethyl tartrate	positive (5.0)	blue
25	Diethyl citrate	positive (5.0)	blue
26	Butyrobetaine (3-carboxypropyl)-trimethyl-ammonium hydroxide inner salt)	positive (10.0)	blue-grey
27	Choline bromide	positive (30.0)	purple
28	[3-(Ethoxycarbonyl)-2-hydroxy-propyl]-trimethyl-ammonium chloride	positive (10.0)	blue-grey
29	(3-Carboxy-2-hydroxypropyl)-trimethylammonium chloride acetate	positive (10.0)	blue-grey
30	Tristearin	negative	
31	1,2-Dimethoxyethane	negative	
32	Cineole (1,8-epoxy-*p*-menthane)	negative	

The several amino acids tested (14–17, Table VI) failed to give positive results. Serine, which contains a hydroxyl group in addition to an amino and carboxyl group, was also negative. Also, the carboxyl group appeared to interfere with the assay since the hydroxy acids (20, 21, Table VI) failed to give positive results though the ethyl esters (24, 25, Table VI) of these hydroxy acids did. Esters and ethers having no other functional groups did not react with the reagents (30–32, Table VI).

The quaternary ammonium compounds (27–29, Table VI) did not require pre-treatment with reagent A (tosylation) to give a positive result because these groups are easily displaced[7] by nucleophiles such as PNBP. Thus, it is possible to differentiate between alcohols (which require the 3-step procedure for assay) and substances carrying such functional groups as aziridine, oxirane, sulfonate, alkyl halides, and quaternary amino as substituents which require an alternative method of assay.

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CHROM. 3895

THE QUANTITATIVE DETERMINATION OF METHYLENEDIOXY COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY–DIRECT DENSITOMETRY

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SUMMARY

A method for the quantitative analysis of methylenedioxy derivatives is described. The method consists of chromatography on thin-layer plates, application of a chromotropic–sulphuric acid spray reagent and analysis of the resultant characteristic purple spots by direct densitometry. Piperonal, piperine, piperonyl butoxide and safrole were chosen as representative compounds and these could be determined in the gamma range.

The methylenedioxy group is present in a large number of natural and synthetic compounds which have found importance as flavouring agents, perfumes, insecticide synergists and physiologically active agents. Numerous analytical techniques for the determination of members of this general class of compounds exist and these have been mentioned in a recent review which lays special emphasis on chromatographic procedures[1].

As part of a programme for the quantitative analysis of methylenedioxy derivatives in foodstuffs, the use of thin-layer chromatography (TLC) coupled with direct densitometry (DD) was investigated. This combination of techniques appeared attractive because a separation or partial clean-up of a mixture can be achieved on the TLC plate, identification is facilitated by the judicious use of R_F values and quantitation can be achieved directly.

Four representative compounds—piperonal, piperine, piperonyl butoxide and safrole were chosen for study. This paper describes a method that can be employed for the quantitative analysis of these and related compounds in the microgram range.

EXPERIMENTAL

Standard solutions

Piperonyl butoxide (Labelled 100%; Niagara Chemical Division, F.M.C. Corp., Middleport, N.Y.), piperonal (m.p. 36–37°; C.A. Aromatics Corp., Floral Park, N.Y.), safrole (b.p. 232–234°; J. T. Baker Chem. Co., Phillipsburg, N.J.), oil of sassafras

Fritzsche Bros., New York) were all used without further purification at a concentration of 1 mg/ml in benzene. Piperine (m.p. 127–129°; Fritzsche Bros., New York) was used as received at the above concentration in methanol–benzene (1:1).

Thin-layer chromatography

TLC plates (20 × 20 cm) were prepared with a Desaga apparatus at a thickness of 250 μ using Adsorbosil-1 (10% binder, Applied Science Labs. Inc., State College, Pa.). The finished plates were air-dried for 0.5 h, activated at 90–100° for 1 h, and stored over silica gel. The plates were all used on the day of preparation.

The chromatographic tanks were lined with 3 mm filter paper and were saturated. Solvents were reagent grade and the systems used were: (A) 15% ethyl acetate in benzene; (B) benzene–*n*-hexane (1:1); (C) 10% benzene in methanol.

The methylenedioxy compounds were applied onto the TLC plates with lambda micropipettes (1 and 5λ, Drummond Scientific Co., Broomall, Pa.) keeping the spots 1.5–2 cm apart and as small as possible. Spot concentrations were 1, 2, 5, 10 and 20λ. Development in the direction of plate preparation was allowed to proceed till the solvent front reached a marker line 15 cm from the origin. The plates were then air-dried and evenly sprayed with chromotropic–sulphuric acid[2] prepared by the careful addition of concentrated sulphuric acid (15 ml) to a solution of sodium chromotropate (1 g) in water (15 ml). Colour development was achieved by heating the TLC plate at 110-120° for 10–30 min depending on the compounds.

Densitometry

A Photovolt Densitometer (Model 520 M; Photovolt Corp., N.Y.) equipped with a motor-driven TLC stage (2.5 in./min), a Varicord 42B recorder (2.0 in./min) and a Search Unit C was employed. The coloured spots were scanned (transmission densitometry, white light) in a direction at right angles to that of solvent development. The aperture length was adjusted by means of black tape so that it was just long enough to encompass the largest spot in any given run; the width was kept constant at 1 mm. The Search Head was adjusted by means of the levelling screw to just clear the upper surface of the chromatoplate. All readings were taken in a darkened room.

Standard curves

Standard curves were prepared for each plate by plotting the average values of the observed peak areas (peak height × the width at one half the peak height) or observed peak heights *vs.* the amount of material applied. Both square grid and full logarithmic graph paper were used (Figs. 2 and 3).

RESULTS AND DISCUSSION

TLC–DD has been used by a number of workers for the analysis of various compounds[3–5]. DALLAS[6] has recently discussed various factors such as layer thickness and development time, which effect the precision in the densitometry of coloured compounds. While these apply, in general, to colourless materials, one of the prerequisites in this case is the development of reagents which react with the compounds in question to give stable coloured spots with no diffusion of colour to the surrounding adsorbent. The intensity must be reproducible and the absorbance of light by the coloured spot

must show a definite relationship to the quantity of material originally present. In this regard, the use of chromotropic–sulphuric acid was found to be suitable both as a spray and for the preparation of self-indicating impregnated plates[2]. This reagent, which yields clearly defined purple spots on a near colourless background when dilute solutions are spotted, is based on the hydrolysis of the methylenedioxy group to yield formaldehyde and the subsequent reaction of the latter with chromotropic acid to form a characteristic purple complex[7]. Although both detection modes may be employed, only the spray method, which is more generally applicable[2], was used in this study. The reagent has a high degree of specificity (see however refs. 2 and 8) and the resultant chromatograms were stable for 3–5 h when stored in the dark. Normally all densitometric readings were taken immediately after colour development.

Due to the possible occurrence of irregular shaped TLC spots such as crescents etc., it was decided to scan the entire coloured spot. Because of a gradation in size (approx. 2.5–7 mm diam.) caused by the twenty-fold concentration range, the slit length was adjusted to just encompass the largest spot in any given run while the slit width was kept constant at 1 mm. The direction of scan was perpendicular to the direction of solvent flow. This procedure was the most convenient and rapid one as it avoided the necessity of and errors in plate repositioning. In addition the analysis of unknown mixtures was facilitated because, by placing several spots of an unknown beside those of the standard, the plate could be scanned entirely in one pass allowing for the construction of a standard curve and the concomitant analysis of the unknown. Fig. 1 illustrates the results of scanning each of the methylenedioxy compounds used in this study.

Each plate was scanned up to ten times in this manner and both peak heights and peak areas were measured and the average values were plotted directly on square grid and full logarithmic graph paper (Figs. 1 and 3). Although the plots of peak area *vs.* concentration approached linearity more closely than those of the peak height plots,

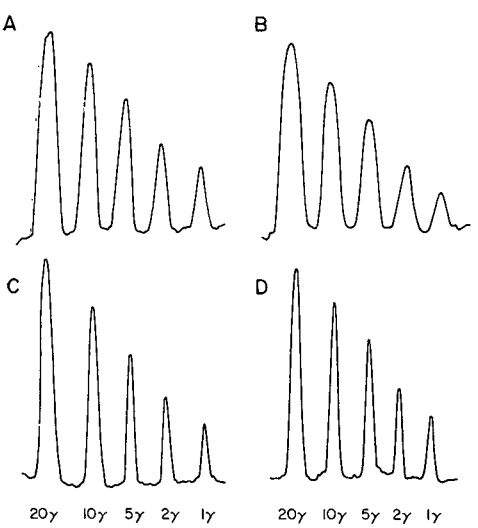
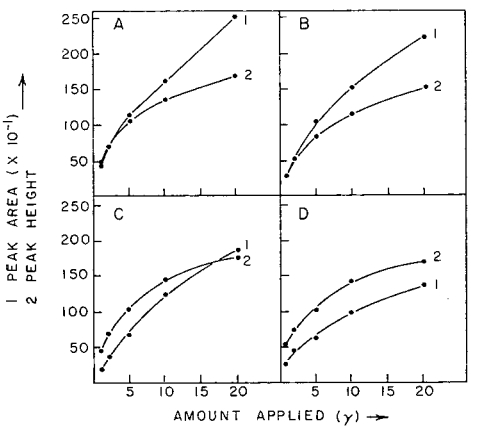


Fig. 1. Densitometric profiles of (A) piperine (plate development in solvent C); (B) safrole (solvent B); (C) piperonal (solvent B); (D) piperonyl butoxide (solvent A).



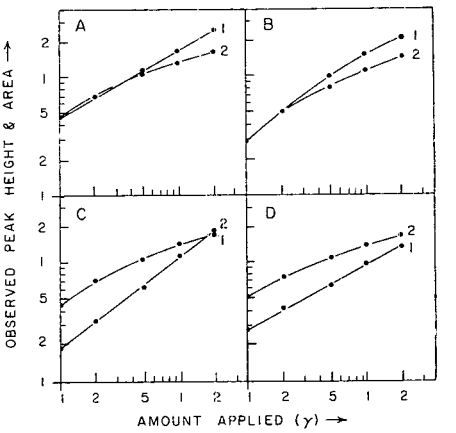


Fig. 2. Standard curves of methylenedioxy derivatives plotted on square grid paper. (A) = Piperine; (B) = safrole; (C) = piperonal; (D) = piperonyl butoxide.

Fig. 3. Standard curves of methylenedioxy derivatives plotted on full logarithmic paper. (A) = Piperine; (B) = safrole; (C) = piperonal; (D) = piperonyl butoxide.

the latter are easier to prepare and there is less error involved in actual measurements. The relative standard deviations pertinent to the determination of the standard curves for the four compounds ranged from 2–4 % for the peak height plots and from 3–7 % for the peak area plots. In practice the choice of which standard curve to employ (square grid or logarithmic plot) would depend on the compound in question.

As an example of this procedure a sample of commercially available oil of sassafras was analysed for its safrole content. Five spots of the standard safrole solution (1–20λ) together with two spots of the essential oil solution (5 and 10λ) were applied onto a TLC plate and the method was carried out as described above. A value of 85 % (± 2%) safrole was obtained using the peak height plot on square grid graph paper. Gas chromatographic analysis of this oil indicated a safrole content of 87 % as determined by disc integration.

In general TLC–DD appears to be of potential use for the quantitative analysis of methylenedioxy derivatives. However, care must be exercised in regard to plate preparation, sample spotting and plate spraying. The procedure, as outlined above, is useful up to a range of 20γ. Larger quantities may be applied to extend the standard curve but varying degrees of colour diffusion[2], depending on the compound in question and the developing solvent will probably limit the useful range to less than 100γ.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. T. B. HAND for technical assistance, Dr. M. R. SAHASRABUDHE for helpful discussions and Fritzsche Bros. and C.A. Aromatics for gifts of chemicals.

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CHROM. 3879

A SIMPLE METHOD FOR THE QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS SEPARATED BY THIN LAYER CHROMATOGRAPHY

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SUMMARY

A rapid, simple and reproducible method for the quantitative determination of phospholipids after separation by thin layer chromatography has been described. The method involves spraying the developed chromatograms with 50 % w/w sulphuric acid, direct mineralization at high temperature and subsequent determination of the liberated inorganic phosphate with HAHN AND LUCKHAUS' reagent. The range of applicability of the method is from 0.05 to 0.5 micromoles of phospholipid phosphate. The method was compared with the generally cited methods used for the same purpose.

INTRODUCTION

Simplicity, good separation ability and quickness characterize thin layer chromatography as an excellent tool for the qualitative examination of phospholipids. Quantitative evaluation of the compounds separated in this class of polar lipids on thin layer chromatograms is, however, a more difficult task. There are two general approaches to the problem[1] of quantitative analysis of phospholipids separated by TLC***:

(1) Elution of the spots from the thin layer plate and subsequent analysis[2–4].

(2) Photodensitometry of the charred chromatogram[5,6].

Both there methods have their disadvantages. Elution is time-consuming (several extractions and centrifugations, evaporation of eluents). On the other hand, elution can be a critical step in this determination because it is not necessarily, in our experience, quantitative.

The photodensitometric method has its limitations which are basically represented by the unequal photoelectric response to the same molar concentrations of different phospholipids. The reason for this discrepancy is the unequal number of carbon atoms in the molecules of individual phospholipids, which are *de facto* deter-

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*** Abbreviations: TLC = thin layer chromatography, P_i = inorganic phosphate, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SP = sphingomyelin, Lyso PC = Lysophosphatidylcholine.

mined by this method. This limitation can be, to some extent, solved by a separate calibration curve for each phospholipid, but the remaining difficulty is the variety of the fatty acids esterified in the naturally occurring phospholipid molecules.

In the following we present a rather simple procedure for the determination of the phospholipids separated by the TLC. The principle of this procedure involves the direct charring of the separated phospholipids on the thin layer plates (with their simultaneous detection) and the subsequent determination of the liberated inorganic phosphate.

The method is simple, reproducible and sufficiently sensitive to be applied to the quantitative determination of phospholipids on TLC plates.

MATERIALS AND METHODS

Standards

Lecithin. This was isolated from egg yolk and purified by passing it first through a column of alumina and than one of silicic acid according to RHODES AND LEA[7]. The final purity of this compound was established from the ester/phosphorus ratio and by thin layer chromatography.

Lysolecithin. This was obtained by the action of cobra venom ("Kobratoxin" — Institute of Sera and Vaccines, Prague) on lecithin according to LONG AND PENNY[8]. After incubation the lysolecithin obtained was treated as described by LONG *et al.*[9] and finally purified by passing through a silicic acid column. The purity of the fractions eluted was monitored by TLC.

Phosphatidylethanolamine. This was obtained and its purity established in the same way as in the case of lecithin. For estimation of the ester/phosphorus ratio we determined the ester moiety by SHAPIRO's[10] method and the phosphorus by the method described by BERENBLUM AND CHAIN[11] as modified by LONG[12].

Reagents

HAHN AND LUCKHAUS reagent[13] was prepared by dissolving 6.85 g sodium molybdate dihydrate and 400 mg hydrazine sulphate in 100 ml of distilled water. To this solution, 100 ml of concentrated sulphuric acid and 500 ml of water were added. After cooling the volume of the solution was adjusted to 1000 ml with water.

Thin layer chromatography

The plates were coated with a layer of silicic acid, 0.5 mm thick. We used Silica Gel CH (5–30 μ), a product of Lachema-Brno with 10 % $CaSO_4$ added. For five plates a slurry of 41 g silica gel in 95 ml distilled water was used. Before use the plates were activated by heating at 115° for half an hour. The developing system was chloroform–methanol–water (65:25:4, v/v).

Mineralization

To obtain phosphorus from phospholipids in the form of inorganic phosphate we ashed the organic matter on the thin layer plates. The developed and dried chromatograms were sprayed with sulphuric acid. Optimal conditions of direct mineralization of thin layer plates were examined and treated statistically. For the evaluation of the influence of temperature, time of mineralization and composition of the spray-

TABLE I

VARIABLES STUDIED IN LATIN SQUARE EXPERIMENT

Factors and units	*Levels*		
Temperature (°C)	I = 180	II = 200	III = 240
Time (min)	1 = 20	2 = 30	3 = 60
Composition of spraying mixture (% of H_2SO_4, w/w)	A = 30	B = 50	C = 70

TABLE II

LATIN SQUARE EXPERIMENTAL DESIGN AND THE PERCENTAGE RECOVERY OF P_i AFTER MINERALIZATION

	I	*II*	*III*	*Percentage recovery*		
1	A	B	C	110.2	99.2	98.2
2	B	C	A	103.2	95.3	106.2
3	C	A	B	96.3	107.2	100.2

ing mixture a Latin square of 3 × 3 terms was used[14]. The three variables studied and their levels and designations are set out in Table I. The combinations of variables actually used can be seen in Table II.

From the statistical evaluation it is evident that only the influence of the levels of spraying reagent composition is significant. The best values were obtained under the following conditions: concentration of spraying mixture 50 % w/w, temperature 180°, time of mineralization 60 min.

Estimation of phosphate

The inorganic orthophosphate obtained after direct mineralization was determined quantitatively using HAHN AND LUCKHAUS' reagent. The black spots from the mineralized plates were scraped off and quantitatively transferred to the centrifuge tubes. 4 ml of distilled water and 1 ml of HAHN AND LUCKHAUS' reagent were added to the tubes. After mixing, the tubes were placed in a boiling water bath for 30 min, during which time the blue colour was developed. After cooling the tubes were centrifuged for 10 min at 3000 r.p.m. to precipitate the silicic acid. Several areas of the same dimensions at different levels were taken as controls. Immediately after centrifugation the optical density of the blue supernatants were measured spectrophotometrically at 700 nm on a CF – 4 single beam spectrophotometer, Optica, Milan, using glass cells with a 1 cm light path. The spectral curve of the blue complex in the range of 300–900 nm is given in Fig. 1.

RESULTS AND DISCUSSION

We analysed samples containing known amounts (0.155, 0.310, and 0.465 μmoles of P_i) of lecithin, lysolecithin and phosphatidylethanolamine, by the method described. The values obtained and the calibration curves are presented in Table III

and Fig. 2. A sample of impure natural lecithin containing some other phospholipids was also analyzed. The results obtained are presented in Table IV. Recovery of phospholipids was 95.3 ± 12.9%. We also studied the reproducibility of our method on a sample of pure lecithin. Results are summarized in Table V. Standard deviations of I, II, III, *i.e.* 0.155, 0.310 and 0.465 μmoles P_i are 13.80, 4.51, and 6.01, respectively.

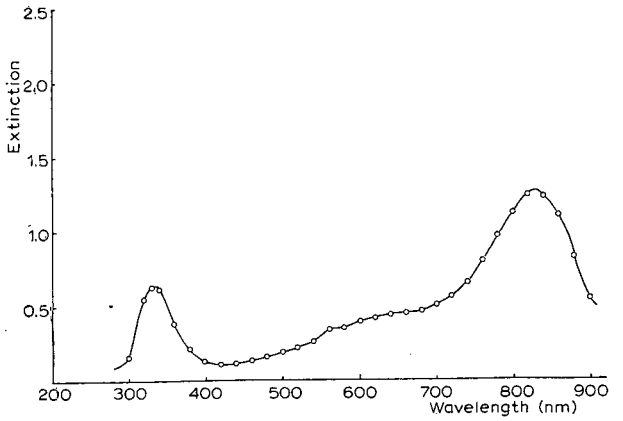

Fig. 1. Spectrum of the blue complex.

TABLE III

VALUES OF P_i OBTAINED BY ANALYSING KNOWN AMOUNTS OF STANDARD PHOSPHOLIPIDS

Every result represents a mean of 10 determinations. I, II, III and IV means 0.000, 0.155, 0.310 and 0.465 μmoles P_i, respectively.

Phospholipid	*Extinction at 700 nm*			
	I	*II*	*III*	*IV*
Lecithin	0.064	0.268	0.466	0.699
Lysolecithin	0.068	0.276	0.465	0.673
Phosphatidylethanolamine	0.060	0.274	0.445	0.707

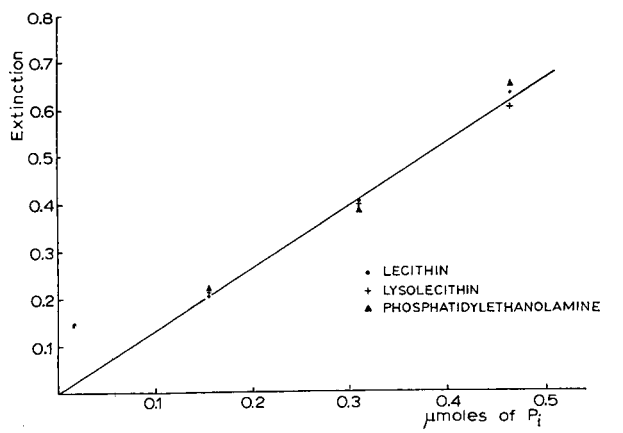

Fig. 2. Calibration curve for lecithin, lysolecithin and phosphatidylethanolamine.

TABLE IV

VALUES OBTAINED BY ANALYSIS OF A PHOSPHOLIPID MIXTURE

(Amount of phospholipids 0.775 μmoles P_i, conditions of TLC as described under MATERIALS AND METHODS.)

No. of estimation	*Micromoles P_i*				
	PE	*PC*	*SP*	*LysoPC*	*Start*
1	0.074	0.577	0.051	0.034	0.010
2	0.068	0.505	0.047	0.026	0.009
3	0.094	0.553	0.071	0.040	0.010
4	0.078	0.579	0.042	0.081	0.000
5	0.087	0.497	0.042	0.043	0.018
6	0.082	0.633	0.028	0.072	0.000
% w/w (average)	10.8	75.3	6.3	6.6	1.0

TABLE V

THE REPRODUCIBILITY OF OUR METHOD

I = 0.155, II = 0.310, III = 0.465 μmoles P_i.

No. of estimation	*Percentages P_i (w/w)*		
	I	*II*	*III*
1	67.3	86.8	90.0
2	76.0	89.0	93.7
3	86.7	89.4	95.6
4	93.5	89.6	96.7
5	94.3	90.2	97.7
6	97.5	95.0	102.5
7	101.5	96.5	104.7
8	103.2	96.7	105.4
9	112.2	98.2	105.6
10	115.5	99.3	107.8
	$\bar{x}$ = 94.77	$\bar{x}$ = 93.07	$\bar{x}$ = 99.97

The differences between experimental and theoretical values are not statistically significant.

As the next step we compared our method with the method described by SKIPSKI *et al.*[2]. These authors repeatedly eluted separated phospholipids from a thin layer chromatogram. The elution was performed with 3 ml and 2 ml of chloroform–methanol–acetic acid–water (25:15:4:2, v/v), 2 ml of methanol and 2 ml of methanol–acetic acid–water (94:1:5, v/v). Finally the eluent was evaporated and the quantity of P_i was determined as in our method. Using their elution procedure we analysed a sample of lecithin in order to obtain a calibration curve. The extinctions for quantities of 0.000, 0.155, 0.310 and 0.465 μmoles of P_i were 0.032, 0.128, 0.287 and 0.507, respectively. The values represent the mean of three determinations.

We also analysed the reproducibility of the method of SKIPSKI *et al.*[2] and obtained the results presented in Table VI.

TABLE VI

THE REPRODUCIBILITY OF THE METHOD DESCRIBED BY SKIPSKI, PETERSON AND BARCLAY[2]
I = 0.155, II = 0.310, III = 0.465 μmoles P_i.

No. of estimation	*Percentages P_i (w/w)*		
	I	*II*	*III*
1	43.8	32.0	53.9
2	47.7	48.3	56.0
3	61.9	53.0	58.1
4	62.6	63.5	62.6
5	63.2	67.7	64.1
6	68.4	68.2	64.6
7	80.7	68.6	66.4
8	84.5	77.0	72.2
9	89.0	77.7	74.0
10	101.6	82.9	87.0
	$\bar{x}$ = 70.34	$\bar{x}$ = 63.89	$\bar{x}$ = 65.89

Standard deviations of I, II, and III, *i.e.* of the results obtained for the concentrations of 0.155, 0.310 and 0.465 μmoles P_i were 18.37, 15.48 and 9.83, respectively. From statistical values it could be seen that the analysis of phospholipids according to our method gives more correct results than elution method described by SKIPSKI *et al.*[2].

To compare our method with other methods often used in the quantitative determination of phospholipids separated by thin layer chromatography[15–19], we mineralized the phospholipids after scraping off the spots visualized by iodine vapour. This mineralization was performed with 70% perchloric acid at 200° for 2 h. After digestion the determination of inorganic phosphate was accomplished using the method described and the reagent of HAHN AND LUCKHAUS. The extinctions obtained with a sample of lecithin according to this method were 0.139, 0.378, 0.494 and 0.663. for quantities of 0.000, 0.155, 0.310 and 0.465 μmoles P_i, respectively. The values of the extinctions represent the mean of three determinations.

It is evident that the extinctions obtained fro the same sample and quantity of lecithin by our method and this one are very close (*cf.* extinctions given in Table III). The only difference is a rather high blank value obtained by the latter method. The further advantage of our method is the omission of the detection of the phospholipids separated on the thin layer plates, which means a shortening of the complete procedure.

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CHROM. 3872

DÜNNSCHICHT-CHROMATOGRAPHISCHE TRENNUNG VON TOMATIDENOL, SOLASODIN UND SOLADULCIDIN BZW. YAMOGENIN, DIOSGENIN UND TIGOGENIN

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(Eingegangen am 15. November 1968)

SUMMARY

The separation of tomatidenol, solasodine and soladulcidine and yamogenin, diosgenin and tigogenin by thin-layer chromatography

A description is given of the separation of tomatidenol, solasodine and soladulcidine and yamogenin, diosgenin and tigogenin by thin-layer chromatography. Use was made of silver nitrate impregnated Silica Gel G.

EINLEITUNG

Bei der Untersuchung von *Solanum dulcamara*-Proben verschiedener Herkunft wurden drei chemische Rassen gefunden. Die Steroidalkaloide Tomatidenol, Solasodin und Soladulcidin sind für die einzelnen Rassen kennzeichnend. Auch sind Mischformen bekannt; diese enthalten mindestens zwei der genannten Alkaloide in etwa gleicher Konzentration[1,2].

Die Untersuchung des Pflanzenmateriales erfolgt meistens durch Analyse der Glycoalkaloide[3–5]. Auch die Auftrennung der Alkaloide selbst kann als Nachweismöglichkeit herangezogen werden[6–8]. Eine gleichzeitige DC Auftrennung von Tomatidenol, Solasodin und Soladulcidin ist bis jetzt noch nicht beschrieben worden. Das Ziel dieser Arbeit war daher, nach Möglichkeiten zu suchen, die eine gleichzeitige, eindeutige DC Auftrennung der genannten Alkaloide erlauben. Der Vorteil, den eine solche DC Trennung der drei wichtigsten *Solanum dulcamara*-Steroidalkaloide bei der Aufarbeitung eines grossen Untersuchungsmateriales mit sich bringt, ist eindeutig.

Die *Solanum dulcamara*-Alkaloide werden von den entsprechenden neutralen Steroidsapogeninen Yamogenin, Diosgenin und Tigogenin begleitet[1,9–11]. Diese wurden daher in die Untersuchungen einbezogen. Über die Trennung dieser Verbindungen wurde ebenfalls schon gearbeitet[7,8,10–12], jedoch eine gleichzeitige DC Auftrennung ist nicht beschrieben.

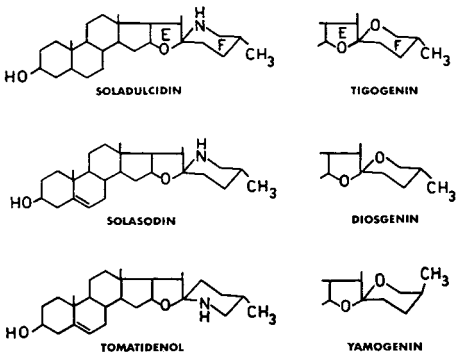

Fig. 1. Strukturformel der untersuchten Steroidalkaloide und Steroidsapogenine.

EXPERIMENTELLER TEIL (soweit nicht aus den Legenden der Fig. 2–5 ersichtlich)

Die uniformen wie die Gradient-Schichten wurden mit dem von STAHL entwickelten Streicher hergestellt (vgl. E. STAHL, in E. STAHL (Herausgeber), *Handbuch der Dünnschicht-Chromatographie*, 2 Aufl., Springer-Verlag, Berlin und Academic Press, New York, 1966, S. 92ff). Schicht: Kieselgel G (Merck) ohne und mit Zusatz von $AgNO_3$; Suspension: 45 g Kieselgel G in 94 ml aqua dest. bzw. $AgNO_3$-Lösung; Schichtdicke: 375 μm; Laufstrecke (in normaler Trogkammer): 15 cm; Temperatur: 22 $\pm$ 2°; rel. Feuchte: 40% $\pm$ 5%; Sprühreagenz: Anisaldehyd–Schwefelsäure mit Phosphormolybdänsäurezusatz (0.5 ml Anisaldehyd, 10 ml Eisessig, 85 ml Methanol, 5 ml konz. Schwefelsäure, 20 g Phosphormolybdänsäure); Erhitzung der besprühten Platten: 110°, 5–10 min.

ERGEBNISSE

Trennung der Steroidalkaloide

Tomatidenol, Solasodin und Soladulcidin (Fig. 1) lassen sich durch DC in normalen Trogkammern unter Kammersättigung auf $AgNO_3$-haltigen uniformen Kieselgel G-Schichten (Fig. 2, rechte Bildhälfte) wie "im Gradient" von $AgNO_3$-haltigem Kieselgel G nach $AgNO_3$-freier Schicht (Fig. 2, Bildmitte) recht gut trennen. Hierbei ist der Chromatographie "im Gradient" von $AgNO_3$-haltiger nach $AgNO_3$-freier Schicht der Vorzug zu geben: Die Substanzflecken sind schärfer umrissen und laufen nicht ineinander. Auf uniformen Schichten tritt oft eine Schwänzung der Substanzflecken auf. Aus der Fig. 2 ist dies deutlich zu erkennen. Die Chromatographie "im Gradient" von $AgNO_3$-freiem nach $AgNO_3$-haltigem Kieselgel (Fig. 2, linke Bildhälfte) bringt dagegen keine Vorteile. Solasodin und Soladulcidin werden nicht getrennt.

Die Durchlauf-Entwicklung in der BN-Kammer (Fig. 3) bringt im Vergleich zur Dreifach-Entwicklung (Fig. 2, Mitte) noch bessere Trennleistungen. Die Flecken sind jedoch diffuser, die Nachweisgrenze liegt infolgedessen etwas höher.

Mit Hilfe der T-Gradient-Technik (Chromatographie "quer zum Gradient") wurde der optimale $AgNO_3$-Gehalt der Schichten für Trennungen in normalen Trogkammern und in der BN-Kammer ermittelt. Optimale Trennleistungen werden in

beiden Kammern bei $AgNO_3$-Gehalten zwischen 4.2 und 6.3 % (bezogen auf Kieselgel) erreicht. Höhere $AgNO_3$-Gehalte wirken sich nachteilig aus: die Trennleistung geht zurück. Das erste Anzeichen einer Trennung von Solasodin und Soladulcidin ist bei $AgNO_3$-Gehalten von etwa 2.1 % erkennbar. Dagegen sind Tomatidenol und Solasodin bzw. Tomatidenol und Soladulcidin bereits auf $AgNO_3$-freier Schicht getrennt.

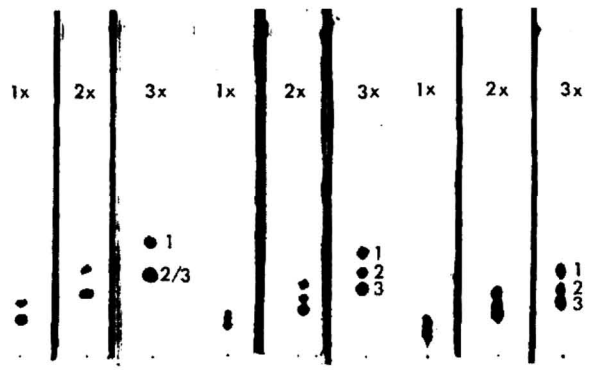

Fig. 2. Trennung der Steroidalkaloide Tomatidenol (1), Soladulcidin (2) und Solasodin (3) in normaler Trogkammer bei Kammersättigung durch Einfach- (1x), Zweifach- (2x) und Dreifach-Entwicklung (3x). Linke Bildhälfte: Chromatographie im $AgNO_3$-Gradient von $AgNO_3$-freiem nach $AgNO_3$-haltigem Kieselgel G ($AgNO_3$-Gehalt maximal 6.3 %); Bildmitte: Chromatographie im $AgNO_3$-Gradient von $AgNO_3$-haltigem ($AgNO_3$-Gehalt maximal 6.3 %) nach $AgNO_3$-freiem Kieselgel G; rechte Bildhälfte: Uniforme $AgNO_3$-haltige Kieselgel G-Schicht ($AgNO_3$-Gehalt 6.3 %); Fliessmittel: Dichlormethan–Methanol (95 + 5); hR_F-Richtwerte bei Dreifach-Entwicklung (im Gradient von $AgNO_3$-haltiger nach $AgNO_3$-freier Schicht): Tomatidenol 31, Soladulcidin 25, Solasodin 21.

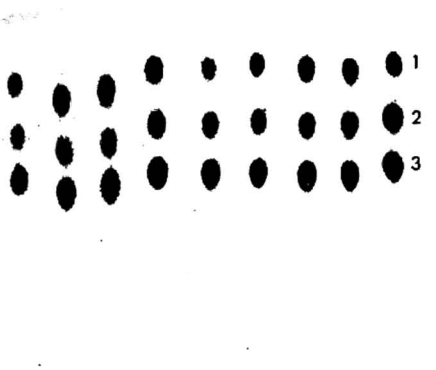

Fig. 3. Durchlauf-Entwicklung der Steroidalkaloide Tomatidenol (1), Soladulcidin (2) und Solasodin (3) in der BN-Kammer. Schicht: uniforme $AgNO_3$-imprägnierte Kieselgel G-Schicht ($AgNO_3$-Gehalt 6.3 %); Fliessmittel: Dichlormethan–Methanol (95 + 5); Laufzeit: 125 min. hR_F-Richtwerte: Tomatidenol 84, Soladulcidin 70, Solasodin 55.

Trennung der Steroidsapogenine

Die Sapogenine Yamogenin, Diosgenin und Tigogenin (Fig. 1)* lassen sich ebenfalls auf mit $AgNO_3$ imprägnierten Kieselgel G-Schichten in normalen Trogkammern bei Dreifach-Entwicklung und in BN-Kammern gut trennen. Für die Chromatographie der neutralen Steroide in Trogkammern (Dreifach-Entwicklung) lassen sich die gleichen Betrachtungen anstellen wie bei der Trennung der Steroidalkaloide unter gleichen Bedingungen. Es hat sich auch hier die Entwicklung "im Gradienten" von $AgNO_3$-haltiger nach $AgNO_3$-freier Schicht als vorteilhaft erwiesen (Fig. 4, Bildmitte): Bei guter Trennung sind die Substanzflecken kompakt rundlich. Gute Trennungen lassen sich auch mit der Durchlauf-Entwicklung (BN-Kammer) erzielen. Fig. 5 zeigt, dass $AgNO_3$-Gehalte von 1.5 % zur Trennung genügen und eine Trennung schon bei einem $AgNO_3$-Gehalt von 1 % erfolgt.

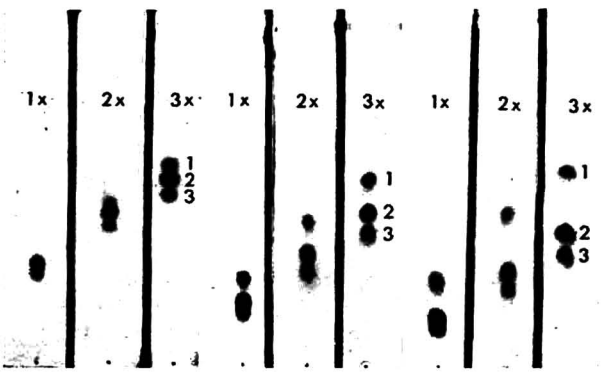

Fig. 4. Trennung der Steroidsapogenine Tigogenin (1), Diosgenin (2) und Yamogenin (3) in normaler Trogkammer bei Kammersättigung durch Einfach- (1x), Zweifach- (2x) und Dreifach-Entwicklung (3x). Linke Bildhälfte: Chromatographie im $AgNO_3$-Gradient von $AgNO_3$-freiem nach $AgNO_3$-haltigem Kieselgel G ($AgNO_3$-Gehalt maximal 2.1 %); Bildmitte: Chromatographie im $AgNO_3$-Gradient von $AgNO_3$-haltigem ($AgNO_3$-Gehalt maximal 2.1%) nach $AgNO_3$-freiem Kieselgel G; rechte Bildhälfte: uniforme $AgNO_3$-haltige Kieselgel G-Schicht ($AgNO_3$-Gehalt 2.1 %); Fliessmittel: Dichlormethan–Aceton (97 + 3); hR_F-Richtwerte bei Dreifach-Entwicklung (im Gradient von $AgNO_3$-haltiger nach $AgNO_3$-freier Schicht): Tigogenin 56, Diosgenin 46, Yamogenin 39.

Sprühreagenz

Als Sprühreagenz wurde Anisaldehyd–Schwefelsäure, dem 20 % Phosphormolybdänsäure zugesetzt waren, verwendet. Dieses Detektionsmittel hat sich vor allem bei $AgNO_3$-haltigen Schichten durch seine hohe Empfindlichkeit gegenüber anderen Nachweisreagentien bewährt: Es lassen sich noch Substanzmengen von 0.2 μg an Steroidalkaloiden bzw. 0.3 μg an Steroidsapogeninen bei Dreifach-Entwicklung und 0.6 μg bei Durchlauf-Entwicklung erfassen.

DISKUSSION

Die Steroidalkaloide Tomatidenol, Solasodin und Soladulcidin und die entsprechenden Steroidsapogenine Yamogenin, Diosgenin und Tigogenin lassen sich

* Der Fa. Merck AG., Darmstadt (Herrn Dr. W. KÜSSNER), danke ich für die Überlassung der neutralen Sapogenine.

ohne Schwierigkeiten auf mit $AgNO_3$ imprägnierten Kieselgel G-Schichten trennen. Als Fliessmittelhauptkomponente hat sich Dichlormethan als am geeignetsten erwiesen. Es übertrifft in seiner Trennleistung das Chloroform. Für die Basen hat sich das Fliessmittel Dichlormethan–Methanol (95 + 5) und für die neutralen Sapogenine Dichlormethan–Aceton (97 + 3; 98 + 2) bewährt. Schon geringfügige Veränderungen im Methanol- bzw. Acetonanteil des Fliessmittels verhindern eine gleichzeitige Auftrennung der drei jeweils untersuchten Verbindungen.

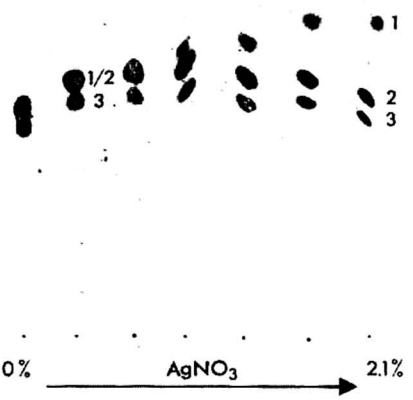

Fig. 5. Durchlauf-Chromatogramm der Steroidsapogenine Tigogenin (1), Diosgenin (2) und Yamogenin (3) in der BN-Kammer unter Anwendung der T-Gradient-Technik; Fliessmittel: Dichlormethan–Aceton (98 + 2); Laufzeit 150 min.

Mit Hilfe der von STAHL entwickelten T-Gradient-Technik (Chromatographie quer zum Gradient) lässt sich schnell entscheiden, welche Sorbtionsmittelkombination am geeignetsten ist, in wie weit und in welcher Konzentration bestimmte Imprägnierungsmittel von Vorteil sind[15–20]. In der vorliegenden Arbeit wurde nun versucht, durch Chromatographie "im Gradienten" selbst bessere Trennleistungen zu erhalten (Fig. 2, Mitte und 4, Mitte). Die hR_F-Werte dieser Trennungen sind etwa die gleichen wie auf uniformen $AgNO_3$-haltigen Schichten. Die Substanzflecken sind jedoch kompakter und daher deutlicher voneinander getrennt. Eine "Schwänzung", die auf uniformen Platten eintritt, konnte nicht beobachtet werden. Es ist zu erwarten, dass sich bei systematischer Untersuchung weitere Anwendungsbeispiele für eine vorteilhafte Verwendung der Chromatographie "im Gradient" finden lassen.

ZUSAMMENFASSUNG

Es wird die Trennung von Tomatidenol, Solasodin und Soladulcidin wie von Yamogenin, Diosgenin und Tigogenin auf silbernitrathaltigen Kieselgel G-Schichten beschrieben. Ferner wird der Vorteil einer Chromatographie "im Gradient" bei der Auftrennung chemisch recht einheitlicher Verbindungen aufgezeigt.

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CHROM. 3867

AN IMPROVED METHOD FOR THIN-LAYER CHROMATOGRAPHY OF NUCLEOTIDE MIXTURES CONTAINING ^{32}P-LABELED ORTHOPHOSPHATE

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(Received November 8th, 1968)

SUMMARY

Development of poly(ethylene)imine cellulose thin layers with phosphate solutions gives improved resolution of complex mixtures of nucleotides. Phosphate development also minimizes the tailing of highly radioactive orthophosphate present in the mixtures and thus facilitates chromatographic analysis of crude acid extracts of phosphate-labeled bacteria. Conditions employing phosphate development are described which give semi-quantitative resolution of the ribonucleoside triphosphate components of such extracts after one-dimensional chromatography as well as two-dimensional systems for quantitative resolution of the major nucleotide components.

INTRODUCTION

Recently a number of procedures for ion-exchange thin-layer chromatographic resolution of complex nucleotide mixtures have been developed (reviews[1,2]). Among these the use of poly(ethylene)imine cellulose anion exchanger (PEI) has been shown by RANDERATH *et al.*[3-5] to be particularly well suited for nucleotide analysis. However with many of the solvent systems affording the best separation of nucleotidic compounds, we have encountered problems with "tailing" of sizeable amounts of $^{32}P_i$** during TLC on PEI cellulose layers. This contamination precludes the application of unidimensional procedures for the analysis of acid extracts of $^{32}P_i$-labeled bacteria without prior removal of the P_i. Furthermore, even after two-dimensional separations, the quantitative estimation of some nucleotides from ^{32}P radioactivity was impaired by the tailing of $^{32}P_i$. We have minimized this problem through the use of ortho-

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** Abbreviations used: Only 5′-nucleotides were employed in this study. AMP = Adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GMP, GDP, GTP = guanosine mono-, di-, and triphosphate; UMP, UDP, UTP = uridine mono-, di-, and triphosphate; CMP, CDP, CTP = cytosine mono-, di-, and triphosphate; ITP = inosine triphosphate; XTP = xanthosine triphosphate; TTP = thymidine triphosphate; dCTP = deoxycytosine triphosphate; dGTP = deoxyguanosine triphosphate; dATP deoxyadenosine triphosphate. P_i = orthophosphate.

phosphate developing solutions and thus obviated the necessity for purifying such extracts free of $^{32}P_i$ prior to chromatography.

In this communication we describe the properties of phosphate chromatography of nucleotides on PEI cellulose thin layers. In addition, two-dimensional systems are described which we feel give improved resolution of the major nucleotide components of soluble bacterial pools.

METHODS

In general the methods employed are described by RANDERATH AND RANDERATH[6]. Poly(ethylene)imine cellulose thin layers on plastic sheets were obtained from Brinkmann Instruments, Inc., Westbury, N. Y. Prior to chromatography the PEI cellulose sheets were soaked in trays containing 1 l distilled water for 30 min and dried at room temperature with a fan. Chromatography was carried out by ascending development (dry start) in closed plexiglass chambers at room temperature.

Phosphate solutions were prepared from analytical grade phosphoric acid and its potassium salts. Nucleoside mono-, di-, and triphosphates were obtained from Sigma Chemical Co., St. Louis, Mo., and Schwarz BioResearch, Inc., Orangeburg, N. Y. Adenosine tetra- and pentaphosphates were purified from a commercial preparation of adenosine tetraphosphate (Sigma Chemical Co.) by preparative TLC and DEAE Sephadex column procedures[7].

Extraction of $^{32}P_i$-labeled acid-soluble materials from bacterial cultures was accomplished by the addition of an equal volume of 2 *M* formic acid[8]. The cell suspension was then held in ice for at least 15 min, and then clarified by centrifugation for 1 min at room temperature in a Beckman microfuge. Aliquots of the cell-free supernatant were applied 3 cm from the lower edge of the chromatogram and fan-dried prior to development. The identification of radioactive materials was confirmed by their co-migration with authentic nucleotides which were added to the extracts. The ^{32}P-labeled materials were visualized by exposing X-ray film to the dried chromatograms for 18 h, and developing the exposed film[8].

Where indicated in the text, nucleotidic materials were removed from the formic acid extracts by the addition of 20 mg of acid-washed Darco G-60 charcoal. After 5 min, the charcoal was removed from the extract by centrifugation. Elution of the nucleotidic materials from the charcoal was accomplished with 4 ml of an ethanol–H_2O–NH_4OH solution (65:35:0.3). The eluate was freed of charcoal by filtration through Celite and concentrated on a warm hot plate under a stream of warm air.

RESULTS

Fig. 1 shows radioautograms of chromatograms developed in one dimension using each of the procedures previously described for two-dimensional resolution of complex mixtures of nucleotides[4,5] as well as the results of chromatography of identical preparations in various concentrations of phosphate, pH 3.4. In each case 2×10^5 c.p.m. of $^{32}P_i$ was spotted at the origin. It may be seen that phosphate development markedly diminishes $^{32}P_i$ tailing, as compared to the other procedures, as well as diminishing the size of the spots. This effect is relatively independent of the phosphate concentration in the range examined.

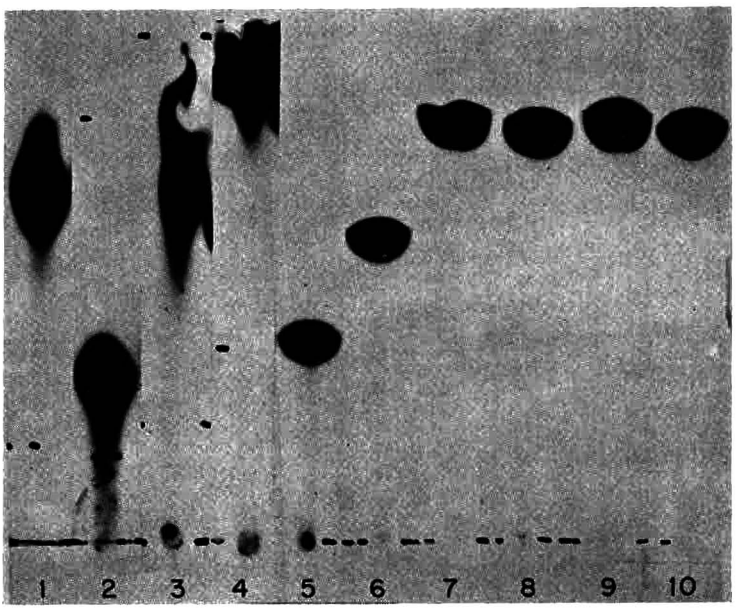

Fig. 1. Comparison of orthophosphate migration and tailing on PEI cellulose thin layers. Approximately 2×10^5 c.p.m. of $^{32}P_i$ were applied to each chromatogram and developed in one dimension under the following conditions: sample 1, step formate[4]; sample 2, step lithium chloride[4]; sample 3, step acetate–lithium chloride[5]; sample 4, step borate–acetate[5]; samples 5 through 10 were chromatographed in KH_2PO_4 (pH 3.4) at 0.25 *M*, 0.5 *M*, 0.75 *M*, 1.0 *M*, 1.25 *M*, and 1.5 *M*, respectively. In each case the chromatograms were developed such that the solvent front was 15–17 cm from the origin.

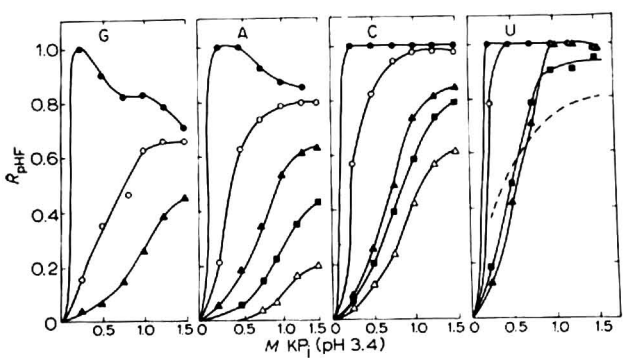

Fig. 2. The effect of potassium phosphate concentration on nucleotide mobilities. Standard nucleotide solutions were chromatographed in phosphate solutions (pH 3.4) at concentrations ranging from 0.25 *M* to 1.5 *M*. The mobilities of the nucleotides are expressed relative to that of the pH front (R_{pHF}). The effect of phosphate concentration on the mobility of the pH front relative to the solvent front (R_F) is the same in all panels but is shown only in panel U as a dashed line. Panel G: GMP, closed circles; GDP, open circles; GTP, closed triangles. Panel A: AMP, closed circles; ADP, open circles; ATP, closed triangles; adenosine tetraphosphate, closed squares; adenosine pentaphosphate, open triangles. Panel C: CMP, closed circles; CDP, open circles; CTP, closed triangles; ITP, closed squares; XTP, open triangles. Panel U: UMP, closed circles; UDP, open circles; UTP, closed triangles, TTP, closed squares.

The effects of phosphate concentration and pH on nucleotide mobilities were explored in order to arrive at a solvent system giving optimal resolution of complex mixtures of nucleotides. Fig. 2 indicates the effect of phosphate concentration (pH 3.4) on the mobilities of several nucleotides found in crude extracts. The mobilities of the compounds are expressed as their fractional movement relative to the pH front (R_{pHF}) since (1) it normalizes nucleotide mobilities to that of inorganic phosphate and (2) no nucleotide moves faster than the pH front. The pH front may be identified by spraying the chromatogram with a suitable pH indicator (Bromocresol Green) or more simply as the second front below the solvent front visible under ultraviolet light. The position of the pH front relative to the solvent front is shown in the right panel of Fig. 2 as a dashed line (- - - -). It is apparent that the mobility of a nucleotide is inversely related to the number of phosphate groups borne by it. The order of increasing mobility exhibited by the nucleoside di- and triphosphates is G, A, C, U, T at 0.75 *M* KH_2PO_4. Notably the mobilities of the purine monophosphates decrease with increasing concentration of phosphate and at high concentrations approach those of the corresponding diphosphates. In contrast, the pyrimidine monophosphates move with the pH front at all concentrations above 1 *M* phosphate.

Fig. 3 shows the effect of pH on nucleotide mobilities in 0.75 *M* potassium phosphate solution. Maximum separation of the four triphosphates is affected in the pH range of 3 to 5. As in Fig. 2, the dashed line (- - - -) describes the mobility of the pH front relative to the solvent front.

The resolution of the nucleotide triphosphates from ^{32}P-labeled compounds not adsorbed to charcoal is shown in Fig. 4. An acid extract of $^{32}P_i$-labeled *E. coli* cells

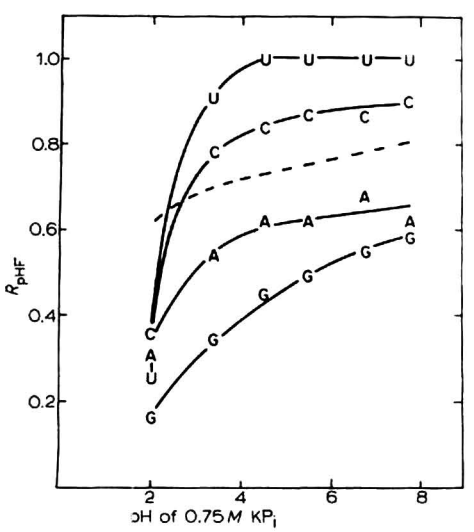

Fig. 3. The effect of pH on nucleotide mobilities. Standard solutions of ribonucleoside triphosphates were chromatographed in 0.75 *M* potassium phosphate solutions at a pH ranging from 2.0 to 7.8. As in Fig. 2 the R_F of the pH front is indicated as a dashed line. The mobilities of the nucleotides relative to the pH front are expressed as R_{pHF} and are plotted against pH. The mobilities of the following nucleotides are shown: G, GTP; A, ATP; C, CTP; U, UTP.

was chromatographed before and after removal of the nucleotidic materials by treatment with charcoal. For comparison the adsorbed nucleotides were eluted from the charcoal and also chromatographed. The four ribonucleoside triphosphates are well resolved from each other and P_i. In this uni-dimensional separation, pyrimidine nucleoside mono- and diphosphates migrate with P_i at the pH front. The area between the pH front and UTP contains AMP, ADP, GMP, and TTP. The material migrating slightly faster than CTP is dCTP. Under the conditions employed dATP and dGTP co-migrate with ATP and GTP respectively. The results obtained with the charcoal-treated extract indicate that the contamination of the triphosphate areas with non-adsorbable $^{32}P_i$-labeled materials is very low; the compound co-migrating with GTP is pyrophosphate.

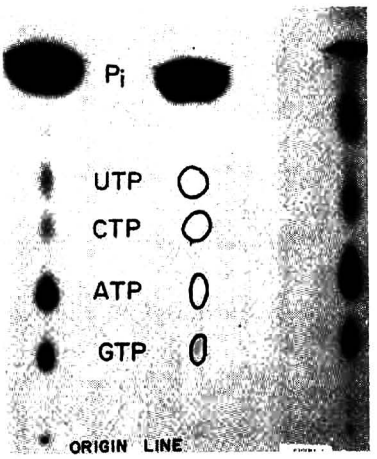

Fig. 4. One-dimensional chromatography of acid extracts of ^{32}P-labeled *E. coli* with 0.85 *M* KH_2PO_4 (pH 3.4). Aliquots of a formic acid extract of ^{32}P-labeled *E. coli* were chromatographed on PEI cellulose thin layers before (left sample) as well as after (middle sample) adsorption of nucleotidic material with charcoal. Chromatography of nucleotides eluted from charcoal, as described in Methods, is shown in the right sample. The mobility of marker nucleotides is indicated in the center panel. The figure shows the radioautogram obtained after 18 h exposure of X-ray film to the chromatogram.

The small discrepancies between the mobility of the triphosphates in the first two columns in Fig. 4 and those shown in Fig. 2 is attributed to the fact that the acid extracts were not adjusted to pH 3.4 before application. The nucleotides eluted from charcoal were applied as an unbuffered neutral solution and consequently their mobilities are the same as those in Fig. 2.

When higher resolution of the pyrimidine triphosphates than can be obtained with the simple one-dimensional system is required, a step formate[4]–0.85 *M* KH_2PO_4 two-dimensional system may be employed to considerable advantage. A typical separation of a crude $^{32}P_i$-labeled extract is shown in Fig. 5a. By first developing the chromatogram with a formate gradient, the nucleoside mono- and diphosphates as well as most of the P_i are resolved from the triphosphates[4]. Separation of the triphosphates is achieved in the second dimension with 0.85 *M* KH_2PO_4, pH 3.4, after removing the formate buffer from the PEI sheets by soaking in methanol for 15 min.

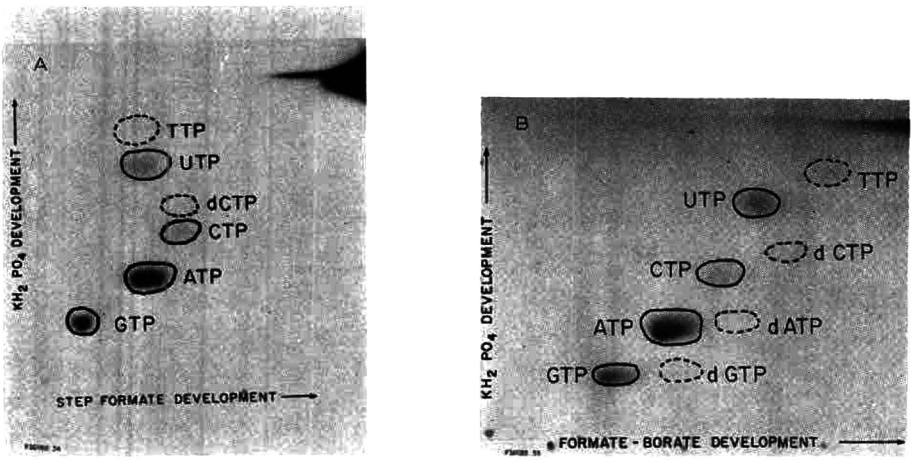

Fig. 5. Two-dimensional chromatography of acid extracts of ^{32}P-labeled *E. coli*. Aliquots of a formic acid extract (similar to that chromatographed in the left panel of Fig. 2) were chromatographed in two dimensions. Panel A: first dimension, step formate followed by soaking in methanol and drying[4]. Panel B: first dimension, 3.3 *M* ammonium formate + 4.2% boric acid (adjusted to pH 7.0 with NH_4OH) followed by soaking in methanol for 5 min, then in distilled water for 15 min, then dried. In panels A and B the second dimension employed 0.85 *M* KH_2PO_4 (pH 3.4). In each case the solvent fronts reached 15–17 cm above the origin in both dimensions.

The purine nucleoside triphosphates are not resolved from the corresponding deoxynucleoside triphosphates. The ammonium borate–acetate–KH_2PO_4 system shown in Fig. 5b affects the separation of all eight ribo- and deoxyribonucleoside triphosphates. However in the latter system, the pyrimidine triphosphates migrate to positions near the nucleoside mono- and diphosphates.

DISCUSSION

Analysis of crude ^{32}P-labeled extracts by thin-layer chromatography is difficult because of excessive tailing of highly radioactive $^{32}P_i$ and the large variety of phosphorylated compounds contained in such extracts. The application of KH_2PO_4 developing solutions minimizes these difficulties. The tailing of P_i observed during phosphate development is sufficiently low and the P_i spot sufficiently compact to allow accurate quantitation of materials moving close to P_i. At the concentrations of potassium phosphate greater than 0.75 *M*, most mono- and diphosphonucleosides as well as the majority of non-charcoal absorbable materials, migrate with P_i at the pH front. Under these conditions it is possible to estimate, semiquantitatively, the four major nucleoside triphosphates in crude extracts using one-dimensional development. Accurate estimation of ribonucleoside triphosphate is easily achieved with either of the two-dimensional systems presented in Fig. 5.

The change in mobilities of the nucleotides with increasing phosphate concentration of the developing solution shown in Fig. 4 is sufficient to mobilize higher homologs of purine nucleotides. Using 1.5 *M* potassium phosphate pH 3.4, we have identified adenosine tetra- and pentaphosphates and guanosine tetraphosphate as contaminants in several commercial preparations of ATP and GTP[7]. The mobiliza-

tion of these highly charged molecules suggests that this system may find application in the separation of oligonucleotides from RNA digests.

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CHROM. 3874

THIN-LAYER CHROMATOGRAPHIC METHOD FOR DETERMINING ANTIOXIDANTS IN POLYETHYLENE AND POLYPROPYLENE FILMS

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SUMMARY

A thin-layer chromatographic method has been developed for determining phenolic type antioxidants in polyethylene and polypropylene films. This paper describes the extraction procedure used for isolating the antioxidants from the polyolefins, the thin-layer chromatographic separation of various antioxidants used industrially, and a quantitative determination of the antioxidant to detect, in our case, 0.02–0.20 % antioxidant by the use of the double beam scanning densitometer. The six antioxidants studied in this investigation are: 4,4′-butylidene(2-*tert.*-butyl-5-methyl)phenol; 4,4′-thiobis(6-*tert.*-butyl-*m*-cresol); pentaerythritol tetrakis(3,5-di-*tert.*-butyl-4-hydroxyhydrocinnamate); 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol); octadecyl (3,5-di-*tert.*-butyl-4-hydroxyphenyl)acetate; 2,6-di-*tert.*-butyl-*p*-cresol.

INTRODUCTION

Antioxidants are used in the food industry to delay or prevent the development of rancidity, and in other industries to enhance the stability of the product. The chemical nature of antioxidants readily permits their analysis, even in low concentrations, by infrared and ultraviolet spectroscopy[1–3]. Distillation[3] and column chromatography[2] have been combined with ultraviolet spectroscopy to determine various combinations of antioxidants; however, the ultraviolet spectra of many additives are often similar, requiring the additional need for a preliminary extraction and infrared analysis to verify the identity.

Antioxidant additives in the plastics industry have given greater durability to polyethylene and polypropylene by decreasing the effect of the polyolefin. The determination of antioxidants in polyethylene and polypropylene[4–6] has been achieved by several authors. However, although the methods are specific in the reported cases, the use of a combination of antioxidants in polyolefins would invalidate the procedures. Although the use of thin-layer chromatography[7–10] is not new for the detection of antioxidants, it was not until SLONAKER AND SIEVERS[11] combined an extraction procedure with thin-layer chromatographic separation that quantitative results were obtained for low concentrations of antioxidants in polyethylene. Since our problem

concerned the separation of various combinations of antioxidants, a thin-layer chromatographic approach was made. This paper is concerned with a different approach toward the isolation of the antioxidants from the polyethylene and polypropylene, which requires only a small sample size (5.0 g) in comparison with the previously reported methods (1 kg); the thin-layer chromatographic separation of various antioxidants, if present in combinations; and a new approach to their quantitative determination whereby, in our case, 0.02–0.20 % antioxidant was detected by use of the double beam scanning densitometer.

APPARATUS

Soxhlet extraction apparatus

The extractor was of medium size, inner diameter 40 mm, top joint 45/50, bottom joint 24/40. The set comes complete with an Allihns condenser, Soxhlet extraction tube and 250-ml flat bottom flask. Extraction thimble: F&S No. 603, size 33 × 94 mm.

Thin layer plates

These were Silica Gel G Uniplates (8 in. × 8 in.) and Silica Gel G Uniplates, reverse phase 5 % Dow Silicone (8 in. × 8 in.). The plates were used as received from the supplier after a short equilibration time in the development solvent vapors.

Capillary pipet

A 20 μl capillary pipet was used.

Chromatographic chamber

This had an inner diameter 10 3/8 in. × 2 3/4 in. × 10 1/4 in., and was supplied with glass cover. The chamber contains an 8 in. × 8 in. sheet of Whatman No. E-17 filter paper to maintain a saturated solvent atmosphere during development.

Chromatography sprayer

Densitometer

This was Joyce Loebl Chromoscan, with 0.0–1.0 optical density wedge, a C-cam 1005 aperture, 5-gain, VS lamp, 620 mμ filter, and a 1:2 gear ratio.

REAGENTS

Extraction solvent

Four volumes of reagent grade *n*-heptane were added to one volume of practical synthetic grade *n*-octane.

Detection system[7,8]

Phosphomolybdic acid (3.0 g) was dissolved in a 100-ml volumetric flask with ethanol and diluted to volume with water. The solution was filtered, if necessary, to remove any solids prior to use. The solution must be prepared fresh every two days.

Development solvent

System A (for identification of antioxidants). 240 volumes of ethanol were added to 80 volumes of distilled water.

System B (for quantitative analysis). 300 volumes of practical grade cyclohexane were added to 6 volumes of reagent grade methanol.

Standard solutions

0.10–0.11 g of the antioxidant to be determined were weighed into a 200-ml volumetric flask and diluted to volume with the extraction solvent. If standards other than 0.10 % (this is based upon a concentration of 5 mg/5 g) are needed, the following equation is used to calculate the percentage antioxidant in the standard.

$$P = \frac{(5)(W_a)}{(W_b)}$$

Where W_a = the sample weight of the antioxidant used to prepare the standard
W_b = sample weight of the polyolefin.

EXTRACTION PROCEDURE

The polyethylene or polypropylene film, 5.0 g (W_b) (precut into strips approximately 1 in. × 1 in.) is weighed into the extraction thimble, 130 ml of the extraction solvent are added to the 250-ml flat bottom flask and a few glass beads; the extraction apparatus is connected and heated to boiling. After 3 h, the system is allowed to cool to room temperature, and the solvent drained into the 250-ml flat bottom flask. The extraction thimble and extraction apparatus are rinsed down with 25–50 ml of the extraction solvent and drained into the 250-ml flask (a turbid solution due to the precipitation of extracted polyolefin may be seen upon cooling; however, this is not uncommon nor should any special attention be taken).

The contents of the 250-ml flask are quantitatively transferred to a 250-ml Pyrex glass beaker (a few glas beads are added to prevent bumping), and the contents concentrated to a 25–30 ml volume by evaporation on a hot plate in a well-ventilated hood. Once the volume is concentrated to 25–30 ml, it is quantitatively transferred to a 50-ml beaker, concentrated to 6–7 ml, quantitatively transferred to a 10-ml volumetric flask, allowed to cool, and diluted to volume with the extraction solvent. If the final solution is turbid or contains precipitate, it is filtered through a Whatman No. 12 filter paper into a 4-dram vial, discarding the first few milliliters; the vial is stoppered.

CHROMATOGRAPHIC PROCEDURE

Two 20-μl aliquots of both the sample and the synthetic standard are applied to the thin-layer plate, the spots are dried with a heat gun, and the chromatograms eluted for 30–40 min in the development solvent. The resulting chromatogram is dried with a heat gun, sprayed with the detection reagent, redried, exposed to ammonium hydroxide vapors, and scanned using the Joyce Loebl Chromoscan. The areas of each zone were calculated by triangulation (area calculated by multiplying

the height times the width at half height). The quantitative results are calculated from the following ratio:

$$\% \text{ antioxidant in the polyolefin} = \frac{A_b \times P}{A_a}$$

Where: A_a = the average numerical area recorded for the synthetic standard;
A_b = the average numerical area recorded for the sample;
P = the actual percentage of the antioxidant present in the synthetic standard based upon the weight of the sample.

DISCUSSION

The direct application of the polyolefin to the thin-layer plate was believed impossible (the polyolefin would hinder migration of the zones making positive identification and interpretation of the resulting chromatogram difficult) and thus a preliminary extraction procedure was incorporated into the method. The extraction procedure described is both simple and straightforward, the only source of error being the extraction of small amounts of the polyolefin. If any of the polyolefin is extracted it must be filtered prior to application to a thin-layer plate, otherwise blockage of the micropipet and a noticeable change in R_F values will be found.

Standard solutions (100 mg/200 ml) of the previously mentioned antioxidants were prepared and 20 μl of each solution were applied to thin-layer plates and eluted with the development solvent. The R_F values of the antioxidants investigated are shown in Table I. System A was used for identification purposes while System B was used for quantitative analysis.

TABLE I

R_F VALUES OF ANTIOXIDANTS

System A: Silica Gel G, reverse phase 5% Dow Silicone; developing solvent, ethanol–water. System B: Silica Gel G Uniplates; developing solvent, cyclohexane–methanol.

Antioxidant	*R_F value*	
	System A	*System B*
4,4′-Butylidenebis(2-*tert.*-butyl-5-methyl)phenol	0.80	0.0
4,4′-Thiobis(6-*tert.*-butyl-*m*-cresol)	0.84	0.0
Pentaerythritol tetrakis(3,5-di-*tert.*-butyl-4-hydroxyhydrocinnamate)	0.60	0.29
2,2′-Methylenebis(4-methyl-6-*tert.*-butylphenol)	0.76	0.34
Octadecyl (3,5-di-*tert.*-butyl-4-hydroxyphenyl)acetate	0.33	0.70
2,6-Di-*tert.*-butyl-*p*-cresol	0.71	0.72

The final aspect of this investigation was concerned with the collection of quantitative data to check both the stability and the precision of the method. The method was made quantitative by spraying the eluted chromatogram with an ethanolic solution of phosphomolybdic acid; the latter yields blue zones for visual detection and visual reflectance measurements on the densitometer. The densitometer measures the blue zone giving a numerical count (integration is internal to the instrument, but not exact for measurement in our case) and a pictorial representation

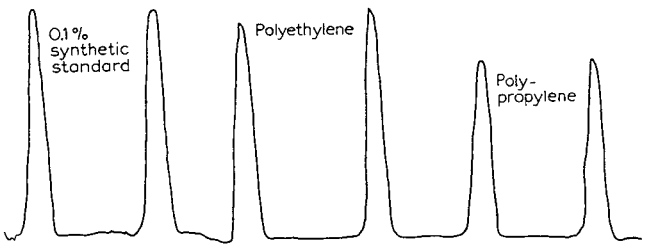

Fig. 1. Chromatographic peak representation using the Joyce Loebl densitometer. Instrument conditions: Joyce Loebl Chromoscan, 0.0–1.0 optical density wedge, C-cam, 5-gain, VS lamp, 620 mμ filter, 1:2 gear ratio. Standard contains 0.1 % 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol).

(Fig. 1) from which the area under the peak can be calculated by triangulation (area calculated by multiplying the height times the width at half height).

In order to check the variables in the thin-layer procedure, solutions of 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol) were prepared in the 0.02–0.20 % range and run according to the chromatographic procedure. A plot (Fig. 2) of the data produces a straight line which passes through the origin. Although this clearly indicates that the percentage antioxidant is directly proportional to the areas recorded, the individual trial runs vary in some cases more than desired. This variation in results can be readily explained from the standpoint of the inability to spray the phosphomolybdic acid solution evenly and thus produce the same colored zones. As will be shown later, the method of spraying the plate will determine to a large degree the depth of the blue color, thus preventing determination of the percentage directly from the calibration curve or from a calibration constant; however, it is important to remember the linear relationship of the concentration to the area. After attempts at eliminating the variation due to the uneven spraying were fruitless, a synthetic standard was introduced into the system. The synthetic standard, which can be prepared in any desired concentration, was spotted alongside the sample, thus exposing both the sample and the standard to the same conditions. The antioxidant content in the polyolefins, prepared to contain approximately 0.1 %, was determined by use of the calibration curve (Fig. 2) and by comparison of the area of the zone from

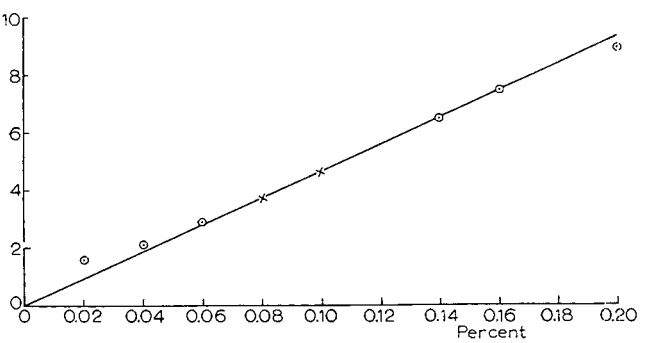

Fig. 2. Calibration curve for 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol).

the sample to that produced by a synthetic standard. The comparison of the results obtained from the two methods is shown in Table II. As can be seen in the data of Table II, the spraying on these particular zones evidently produced darker zones than those tabulated for the calibration curve, thus yielding high results and showing the need for the use of a synthetic standard.

TABLE II

COMPARISON OF RESULTS

Sample	% *A*[a] *by ratio of a synthetic standard*	% *A*[a] *by calibration curve*
Polyethylene	0.095, 0.094, 0.096, 0.092	0.172, 0.163, 0.173, 0.156
Polypropylene	0.084, 0.073, 0.086, 0.080	0.146, 0.135, 0.153, 0.136

[a] 2,2′-Methylenebis(4-methyl-6-*tert.*-butylphenol).

RESULTS AND CONCLUSION

In order to show the validity of the extraction in the thin-layer chromatographic procedures, samples of polyethylene and polypropylene were blended with approximately 0.10 % 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol) and the analysis run according to the recommended method outlined in this paper. The results of these experiments, tested over a 3-day period, along with ultraviolet spectrophotometric data, are shown in Table III. Although there are minor differences in the results of the methods, the thin-layer procedure shows the presence of an additional zone, attributed to the presence of 2,6-di-*tert.*-butyl-*p*-cresol. The presence of this antioxidant, although not determined by the thin-layer chromatographic procedure, could interfere with the ultraviolet procedure thus giving higher results. The extraction procedure, the thin-layer chromatographic procedure, and the quantitative data tabulated by the use of the double beam scanning densitometer offer a new yet simple approach to the determination of various antioxidants in polyethylene and polypropylene. The precision of the method is 10 %, which at low concentration can be tolerated; however, it was not the purpose of this investigation to vary sample size in order to enhance the precision values.

TABLE III

VALIDITY OF THE RECOMMENDED METHOD

Sample	% *Antioxidant*[a]				
	1st day	*2nd day*	*3rd day*	*Average*	*By U.V. analysis*
Polyethylene	0.095, 0.094 0.096, 0.092	0.103, 0.100	0.087, 0.089 0.087	0.094	0.102, 0.105
Polypropylene	0.080, 0.084 0.073, 0.086	0.079, 0.078	0.078, 0.078 0.079	0.079	0.083, 0.083

[a] The antioxidant in this analysis is 2,2′-methylenebis(4-methyl-6-*tert.*-butylphenol).

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CHROM. 3887

INDOLES AND AUXINS

II. NON-DESTRUCTIVE DETECTION OF INDOLES BY ELECTRON ACCEPTORS*

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SUMMARY

The color reactions of several electron acceptors with 48 indoles and related compounds have been studied. The advantages of these complexing spray reagents are that they: (i) are non-destructive (*i.e.* indoles can usually be regenerated); (ii) give an indication of the type of ring substitution (electron-donating or -withdrawing groups); (iii) react with 2,3-disubstituted indoles. 2,4,7-Trinitro-9-fluorenone is the generally most useful reagent. These complexing agents are less sensitive and less specific than other chromogenic reagents for indoles.

INTRODUCTION

Several spray reagents are available for the detection of indole derivatives on paper and thin-layer chromatograms (*cf.* refs. 1–3). These color reagents are sensitive, relatively specific and give a variety of colors with different indoles. For the detection and further characterization of natural or unknown indoles chromogenic sprays presently used have three major disadvantages: (i) the compound in question is chemically changed, usually in an unknown way *i.e.* regeneration of the original indole is impossible; (ii) there is no simple and logical correlation between the structure of the indole and the color produced; and (iii) 2,3-disubstituted indoles cannot be detected easily because color formation usually depends on a coupling reaction of the chromogenic reagent with either the α or β position of the indole ring system.

Indoles have electron-donor properties in charge-transfer complexes (MILLIÉ *et al.*[4] and references cited therein) and highly colored spots are produced when indoles are sprayed with solutions of electron acceptors on paper or thin-layer plates. This property of indoles has not been exploited widely. 2,4,7-Trinitro-9-fluorenone was used as a spot test reagent for the detection of six indoles[5] and recently various electron acceptors have been used to visualize peptides containing tryptophan[6].

A number of colored electron-acceptor complexes of biologically important

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indoles have been studied, mainly in solution (*cf.* refs. 7 and 8) and the properties and usefulness of pure, crystalline complexes of indole compounds are being studied in this laboratory[9]. General references on the chemistry of acceptor–donor complexes are available[7,10–14].

Electron acceptors are a useful addition to presently used indole reagents. The detection of indoles via complex is advantageous with regard to the three points mentioned above.

EXPERIMENTAL

Indoles

The majority of the indole derivatives used in this investigation were commercial samples purchased from the following companies: Aldrich, Calbiochem, Eastman, K & K, Koch-Light, Mann, Regis and Sigma. Ascorbigen A[15], 3-indole acetyl-N-L-aspartic acid[16], 3-indoleacetyl-N$^{\varepsilon}$-L-lysine[17] and N-acetylskatole[18] were prepared by methods described in the literature. Glucobrassicin was a natural sample.

Complexing agents

The following electron acceptors were used (commercial source indicated in parentheses): 1,2,4,5-benzenetetracarboxylic acid dianhydride, tetracyanoethylene, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 4-nitrophthalic acid (Aldrich); chloranil, iodine, maleic anhydride (Anachemia); 2,4,7-trinitro-9-fluorenone, picric acid (Eastman); 2,4-dinitrobenzenesulfonic acid (K & K); 1,3,5-trinitrobenzene and 1-fluoro-2,4-dinitrobenzene (Matheson Coleman & Bell).

Color development

Indoles (10 μg, unless otherwise indicated) in methanolic or aqueous solutions were applied to silica thin-layer sheets and sprayed with 1 % solutions of the electron acceptor in chloroform or acetone. Iodine was used at very low concentration in the vapor phase. The indoles spotted on thin-layer sheets were exposed for 1–2 sec.

Chromatography

Eastman Chromagram sheets (K301R2) were used for chromatography throughout this investigation except where indicated. R_F values are not recorded when in agreement with published data[1,17]. Solvents were: (A) chloroform–methanol–acetic acid (75:20:5), (B) benzene–dioxane (55:45), (C) chloroform–methanol–carbon tetrachloride (50:40:10), (D) isopropanol–34 % ammonia–water (85:5:15), (E) ethanol–water (80:20), and (F) 1-propanol–34 % ammonia (70:30).

3-Indoleacetic acid, 3-indoleacetamide and 3-indoleacetonitrile were chromatographed on plates coated with (i) Silica Gel G (Merck), (ii) Silica Gel G impregnated with 0.2 % 2,4,7-trinitro-9-fluorenone (III)[19] and (iii) Silica Gel G with 3 % III added to the developing solvent[6]. Solvents A–E were used to develop the chromatograms.

Regeneration of indoles

After exposure to iodine. 3-Indoleacetic acid (1 mg) and 3-indoleacetamide (1 mg) were streaked on the base of a 20 × 20 cm thin-layer plate (Silica Gel G (Merck), 0.5 mm thickness) and the chromatogram was developed in solvent D. The plate was

exposed to iodine vapor, the position of the bands marked and the plate immediately placed in a desiccator over potassium hydroxide and evacuated (>0.2 mm) for 2 h. After this time, the brown color had almost completely disappeared. In a separate experiment, samples (10 μg) of tryptamine, 3-indoleacetic acid, 3-indoleacetamide and 3-indoleacetyl-N$^{\varepsilon}$-L-lysine were spotted on silica sheets, exposed to iodine vapor, marked and immediately sprayed with a solution of sodium thiosulfate (0.5 %) in ethanol–water (70:30). Silica from the marked areas in both experiments was removed, extracted with methanol or water and portions of these solutions spotted on silica sheets with corresponding standards and chromatographed in solvents C, D and F.

From complexes with 2,4,7-trinitro-9-fluorenone. The indoles were spotted on Eastman Chromagram sheets: silica (K301R2), alumina (6062), Reeve Angel paper loaded with Amberlite IRC-50 (weak acid, H^+ form) or Amberlite IR-4B (weak base, OH^- form). A 5–10 mm wide strip along the starting line was sprayed with the electron acceptor and the sheet developed in either chloroform, benzene, benzene–hexane (9:1) or ethanol containing 5, 25 and 50 % water. The pure indole compound was eluted with methanol or methanol-34 % ammonia (95:5) after the area had been cut out, scraped off or cut into narrow strips for chromatographic elution. For the decomposition of complexes by solvation, the colored spots were removed from the plate and the silica shaken with water of different pH and dichloromethane or ethyl acetate. Ehrlich's reagent (for indoles) and 5-aminoindole (for electron acceptors) was used to locate the compounds in fractions and on chromatograms. The purity of all regenerated indoles was ascertained by chromatographic comparison with pure standards.

RESULTS AND DISCUSSION

The colors obtained from indoles after spraying with electron acceptors are shown in Table I (natural indoles and related compounds) and Table II (ring-substituted indoles and compounds chemically related to indole). The colored spots on silica sheets showed no fluorescence under long- or short-wave ultraviolet light. Generally, the colors changed little after the third day and were stable for several weeks in a laboratory atmosphere.

In addition to the electron acceptors listed in the tables 2,4-dinitrobenzenesulfonic acid, 1,2,4,5-benzenetetracarboxylic acid dianhydride, 6-nitrophthalic acid and 1-fluoro-2,4-dinitrobenzene were used as spray reagents on some indoles successfully.

Color differentiation, sensitivity and specificity

Structurally similar compounds *i.e.* indoles with the same substitution pattern (*e.g.* 3-indole–CH_2–) gave similar colors with individual spray reagents in a wide variety of shades. The color formed with ring-substituted indoles shows a clear and consistent correlation with the electron-donor or -acceptor properties of the substituent. With four complexing agents the color varies from yellow to organe, red and brown to purple and violet when to the substituent is changed from strongly electron withdrawing to electron donating (–NO_2, –CN; halogen; H; –CH_3; –OH, –NH_2) in position 5 of the indole ring (Table II; also serotonin and 5-hydroxy-3-indoleacetic acid in Table I). Electron-withdrawing groups such as –CHO and –COOH (position 3; Table I) and –CO–CH_3 (on the nitrogen; Table II) have the expected effect on the color produced with complexing reagents. The increase in complex stability with

TABLE I

COLOR[a] REACTIONS OF NATURAL INDOLES AND RELATED COMPOUNDS WITH ELECTRON ACCEPTORS

Indole	*Color development with electron acceptor*[b]													
	I		*II*		*III*		*IV*		*V*		*VI*		*VII*	
3-Indoleacetic acid	V	VBr	LGr	Br	Br	RBr	LO	LOBr	Br	Br	BrR	RBr	Bl	Br
3-Indoleacetamide	V	VBr	LGr	BrOl	Br	OBr	LO	YBr	LBr	LBr	BrR	RBr	Bl	LBr
3-Indoleacetonitrile	Br	DBr	Ol	OBr	R	BkR	Y	OY	Br	Br	O	Br	V	RO
3-Indoleacetic acid ethyl ester	O	GyO	LGr	GrY	Br	RBr	O	OY	Y	Br	O	P	Bu	LO
3-Indoleacetaldehyde	V	Br	LR	Br	O	VBr	O	GyO	Y	Br	LO	VBr	Ma	VBr
3-Indolepyruvic acid	Br	Br	Gr	Br	Br	RBr	O	GyO	Br	GyBr	Br	Br	V	VBr
3-Indolelactic acid	Br	GyBr	LGr	GyBr	Br	RBr	O	GyO	Gy	Gy	R	LRBr	Gy	GyBr
3-Indolepropionic acid	V	VBr	Gr	GyBr	DBr	BrV	O	O	Br	BrGy	R	GyV	V	VBr
3-Indolebutyric acid	LV	VBr	Gr	GyBr	DBr	BrV	DO	DO	Gy	DGy	R	GyV	DV	RV
3-Indoleglyoxylic acid	GyPk	GyPk	GyGr	Gy	O	Br	Y	YBr	Y	YBr	LGrBr	RBr	LY	LYBr
3-Indoleglycolic acid	GyV	LBr	Gr	Gr	Br	PkBr	Y	LYBr	P	VBr	GyPk	PkBr	O	YBr
3-Indolesuccinic acid	GyV	VGy	Ol	VGy	Br	PkBr	Gy	LOlBr	Gy	GyBr	GyBr	VBr	Y	YBr
3-Indoleacetone	Br	DRBr	Gr	GyRBr	Br	RBr	O	OBr	Br	Br	Br	Br	Y	YBr
3-Indolecarbinol	RBr	GyRBr	Bd	Bd	Br	DBr	O	Br	Ma	Ma	Bd	Bd	O	RBr
Gramine	V	V	LY	PkBr	Br	DRBr	O	DO	LY	LOBr	Y	RO	O	O
Tryptophol	LV	LV	LGr	OlY	Br	BrV	O	O	Bu	BuBl	O	OGy	DBu	P
Tryptophan	V	VBr	Gr	BrGy	Gy	BlV	LO	LO	LY	GyOl	O	GyPk	O	GyV
Tryptamine	Br	GyBr	Gr	BrGy	Gy	BlV	O	O	LY	OlGy	O	GyO	Bu	GyV
Serotonin	GyV	VGy	OlGy	BrGy	P	BrV	P	GyBr	Gy	BrGy	GyBr	BrGy	Gy	GyBr
5-Hydroxy-3-indoleacetic acid	P	DBrV	DGy	DGy	Gy	GyV	O	DBr	Br	BrBl	R	BrBl	Bu	DBr
3-Indolecarboxaldehyde	V	PkV	LR	LBr	Y	DY	LY	LO	Y	YGr	LO	LBr	Y	Y
3-Indolecarboxylic acid	V	V	LBr	LBr	DY	DY	LY	Y	Pk	Pk	O	RBr	O	O
3-Indoleacetyl-N-L-aspartic acid	V	Br	LGr	LBr	LBr	RBr	LO	LO	LO	LOlBr	R	RBr	O	O
3-Indoleacetyl-N$^{\epsilon}$-L-lysine	LV	LV	LY	LBr	LBr	LRBr	LO	LO	Y	LOlBr	O	LBr	O	LPk
3-Indoleacrylic acid	Bu	BlV	P	PBr	DBr	RBr	O	Br	Br	Br	R	RBr	Bd	DBd
Indican (indoxyl sulfate)	BuGy	GyV	Gr	GyGr	LBr	LBr	LBr	Y	P	LBr	GyV	VBr	Gy	Y
Ascorbigen	WBr	WBr	LGr	LOlBr	LBr	PkBr	LO	YBr	LPk	LOl	Y	Y	LBu	Y
Glucobrassicin	WBr	WBrV	LGr	LPk	LBr	LBr	LO	YBr	LY	LY	LY	LY	LY	LY

[a] Color abbreviations: Bd = burgundy; Bk = brick; Bl = black; Br = brown; Bu = blue; Ch = charcoal; Gr = green; Gy = grey; Ma = maroon; Mv = mauve; O = orange; Ol = olive; P = purple; Pk = pink; R = red; V = violet; Y = yellow; L = light; D = dark; W = weak; - = no color development.

[b] The color was read overnight (first column for each electron acceptor) and after three days (second column). I = Chloranil; II = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, reddish background; III = 2,4,7-trinitro-9-fluorenone, brownish background after several days; IV = 1,3,5-trinitrobenzene; V = maleic anhydride; VI = picric acid, yellow background; VII = tetracyanoethylene, light yellow background.

TABLE II

COLOR[a] REACTIONS OF RING-SUBSTITUTED INDOLES AND COMPOUNDS RELATED TO INDOLE

Compound	*Color development with electron acceptor*[b]							
	III		*IV*		*V*		*VII*	
Indole	BkR	OR	Y	Y	LY	Br	DO	DO
Skatole	DBr	VBr	O	O	P	P	O	YO
2,3-Dimethylindole	P	PV	BkR	LBr	R	RDBr	Bu	Br
5-Methylindole	O	BrO	Y	Y	Mv	Mv	R	RO
5-Hydroxyindole	Br	VBr	LO	Br	LY	VBr	RBr	RBr
5-Aminoindole	P	BlV	Br	Br	Y	DBr	DBr	Br
5-Fluoroindole	O	YO	Y	LY	Y	RBr	O	YO
5-Chloroindole	DO	O	Y	GyY	LY	RBr	DO	YO
5-Bromoindole	O	O	LY	LY	LY	OBr	O	YO
5-Cyanoindole	O	YO	LY	LY	O	YO	O	Y
5-Nitroindole	O	YO	LY	LY	Y	YO	O	Y
N-Acetylskatole	Y	O	LY	Y	—	—	LY	—
2,3-Diphenylindole	Gy	Gy	Br	Br	LY	LO	LY	Y
Oxindole	Y	DY	Ch	OlBr	—	WLY	O	WLY
Isatin	Y	RBr	Y	Y	Y	Y	O	O
Indoxyl acetate	Br	DBr	Br	Br	P	P	P	P
Indoline	P	BrGyV	Mv	DBr	O	BlBr	R+Gy	DBr
2,3,3-Trimethylindolenine	P	BuBl	Gy	Br	Mv	RBr	R	RO
Pyrrole	GyBr	GyBr	Br	Br	Br	Br	Br	Br
Carbazole	BkR	BkR	Y	Y	Pk	PkGy	—	GyBr

[a,b] See footnotes to Table I.

increasing electron-donor properties of the indole is reflected in these color changes[14].

The influence of a substituent's position on the color was not investigated. With the exception of some methyl derivatives, all substituents were tested in one position of the indole ring only. From theoretical calculations and charge-transfer spectra, however, additional color changes with some substituents can be expected[4,20,21]. The shift to violet colors with methyl groups is more noticeable in position 3 than 5 with most complexes. 2,3-Dimethyl substitution gives a much larger change. This could be due to localized electron-donor abilities of indoles[22] (positions 2 and 3) and the lack of long-range electronic effects of the methyl group.

The limits of detection for indoles are higher with these complexing reagents than with most popular indole spray reagents[2,23]. Limits of detection for 3-indoleacetic acid with electron acceptors I–VII are as follows: I, II and III, > 1 μg; IV and V, 1–2 μg; VI and VII ~ 1 μg. The same order of magnitude is evident for most other indoles by qualitative observation. Reagents I, II and III are the most sensitive in all cases.

Electron acceptors I–VII give color reactions with 2,3-disubstituted indoles, compounds that cannot be detected satisfactorily with most indole sprays. 2,3-Dimethylindole gives intense brown, red and violet colors with all seven complexing reagents at 10 μg. At the same concentration only faintly blue coloration is observed with Ehrlich's reagent and no color with Salkowski's reagent.

A variety of compounds other than indoles give colors with complexing reagents I–VII. Some of these electron acceptors are actually used as spray or streak reagent for other compounds[5,6,24–27]. All good electron donors such as amines, some hetero-

cyclic and aromatic compounds (particularly when polycyclic or substituted by one or more hydroxyl or amino groups) would be expected to give colors. Spots (10 μg) of L-tyrosine, L-histidine, L-phenylalanine, L-lysine and 1-naphthylacetic acid were sprayed with complexes I–VII. I–IV, VI and VII gave colored spots of lower intensity than with indoles. Maleic anhydride (V) was an exception; no color at all was produced.

Although iodine is widely used as a non-specific reagent, no color was produced when the five compounds mentioned above were exposed to iodine vapor at low concentration for 1–2 sec.

In view of the low specificity, it is advisable to preclassify *e.g.* natural extracts with conventional indole spray reagents first or ascertain the indole structure after decomposition of the complex by ultraviolet[28] or luminescence (for references see ref. 29) spectroscopy.

Non-destructive detection and regeneration of indoles from complexes

Iodine as a general staining reagent for many types of organic compounds is in common use. It has also been suggested as non-destructive color reagent for thin-layer and paper chromatograms[30]. The mechanism of color formation varies with different types of compounds, the color formed with indoles is undoubtedly due to complex formation[31].

When several indoles, on thin-layer chromatograms, were exposed to iodine vapor at low concentration for 1–2 sec, well defined brown spots were formed on a colorless background. The color disappeared completely when immediately sprayed with sodium thiosulfate solution and only a faint brown color remained when the chromatograms were evacuated over potassium hydroxide. Thin-layer chromatograms of the material extracted from these spots showed that only unchanged starting material was present in both cases.

2,4,7-Trinitro-9-fluorenone (III) was chosen as an electron acceptor for studying the recovery of indoles from complexes because: (i) it is one of the most sensitive complexing reagents; (ii) in preliminary experiments it was shown to be the most suitable of all compounds I–VII for chromatographic separation from a variety of indoles; (iii) it is very insoluble in aqueous ethanol, III precipitates almost quantitatively when the complex, extracted from silica, is heated in 50–75 % ethanol; and (iv) although aromatic nitro compounds are reactive under certain circumstances[32], permanent chemical change of indoles is much less likely than with some of the other, more reactive electron acceptors; *e.g.* tetracyanoethylene is known to react with indoles resulting in sigma bond formation[33].

Complexes of III with indoles could not be clearly separated by chromatography on activated alumina thin-layer chromatograms with benzene, although this would be expected because of the affinity of this electron acceptor for alumina. Complexes of aromatic hydrocarbons and III *e.g.* can easily be separated by washing the hydrocarbon through an alumina column. 2,4,7-Trinitro-9-fluorenone remains on the top of the column in this case[34]. On the alumina thin-layer sheets used (pH 9) III travels with an R_F value of $\sim$ 0.8 in benzene. Even chromatography with aqueous ethanol is impractical, most of the complexing reagent remains on the starting line but a small amount seems to travel in complexed form.

One representative each of an acidic (3-indoleacetic acid; IAA), basic (tryptamine; trypt.) neutral (3-indoleacetamide; IAM) and water-soluble (3-indoleacetyl-

N$^{\varepsilon}$-L-lysine; IAA-lys.) indole was used for chromatographic separation from their 2,4,7-trinitro-9-fluorenone complex. IAA was quantitatively separated from III on paper loaded with anion-exchange resin. IAA remains on the starting line and can be extracted (5 % ammonia in methanol) after the paper has been developed and III thereby removed from the complex with benzene–hexane (9:1). (IAA streaks in higher concentration.) Similarly trypt. can be recovered from the starting spot (5 % ammonia in methanol) after the complex has been irrigated with chloroform on paper loaded with cation exchange resin. IAM also remains on the starting line when the complex is chromatographed on silica with benzene. IAA-lys. can be eluted from the complex with 50 % ethanol, in this case III remains on the origin. Most acidic, basic and water-soluble indoles are expected to behave similarly to the compounds tested. Some neutral indoles will probably require different systems.

Most indoles can easily be regenerated from complexes by solvation. Use was made of POWELL's[35] separation scheme for acidic, basic, neutral and water-soluble indoles. The compounds mentioned above were used and in some cases ethyl acetate was found to be preferable to dichloromethane. The complexing agent III remained with the neutral fraction (IAM), it had to be separated by the chromatographic method described above. Complexes of IAA, trypt. and IAA-lys. with III were also decomposed individually by shaking with ethyl acetate and water of the appropriate pH.

Decomposition of complexes is not necessary in some instances, mass spectra of more volatile indoles can be obtained on the complex directly[9,36].

Chromatography on layers treated with complexing reagent

Silica impregnated with aromatic nitro compounds proved successful for separating aromatic hydrocarbons[19,37,38]; no advantage for the separation of indoles over regular thin-layer chromatography was found in this investigation. On treated plates, spots were more diffuse and R_F values similar to untreated plates with solvents A–E. Addition of 3 % complexing reagent to the developing solvent[6] also showed no advantages.

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CHROM. 3893

PAPER CHROMATOGRAPHIC DETECTION AND COLORIMETRIC DETERMINATION OF SOME 5-O-SUBSTITUTED TRYPTAMINES (3-(2-AMINOETHYL)INDOLES), UTILIZING THE FORMATION OF XANTHYLIUM SALTS*

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SUMMARY

A method is described which allows the rapid and precise quantitative determination, either directly in expressed plant juice, or in crude alkaloid extracts of 5-hydroxytryptamine (serotonin), N,N-dimethyl-5-hydroxytryptamine (bufotenine), 5-methoxytryptamine, and N,N-dimethyl-5-methoxytryptamine in the presence of tryptophan, tryptamine, N,N-dimethyl-5-methyltryptamine, N,N-dimethyltryptamine, 3-(2-dimethylaminoethyl)-indole (gramine), *p*-(2-dimethylaminoethyl)-phenol (hordenine), or 3-indolylacetic acid. The absorption spectra, effect of variable concentrations in the composition of the developing reagent, and conditions for the determination are reported. The concentrations of N,N-dimethyl-5-methoxytryptamine found in *Phalaris tuberosa* leaves are given.

INTRODUCTION

Indolealkylamines are widely distributed in the plant kingdom[1]. GALLAGHER AND KOCH[2] recently implicated certain of these biogenic amines in a neurological syndrome of grazing animals. The possible involvement of indolealkylamines in the toxicity of range grasses precipitated our investigation of the effects of nutritional and environmental factors on the occurrence and biosynthesis of these amines in range plants. The more toxic of the tryptamines of *Phalaris tuberosa* (Hardinggrass) have been reported to be the 5-O-substituted tryptamine derivatives[2]. Methods for the quantitative determination of these compounds were the primary requirement for subsequent investigation of factors affecting the formation and accumulation of these alkaloids in plants.

One of the many color reactions of indoles is that with xanthydrol. Several methods have been proposed for determination of some tryptamine derivatives by formation of colored xanthylindole salts[3–5]. These methods were tested, but were not entirely suitable. Some of the procedures could not be replicated. Most of the methods investigated did not include the several tryptamines of interest. A method based on

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modifications of these procedures, combining paper chromatography and colorimetry, was developed and tested on ten indole compounds. All the 5-O-substituted tryptamines studied formed xanthylindole salts which had the same absorption maximum. The color formed was proportional to the concentration of the tryptamines. These could be quantitatively determined rapidly and precisely. The method allows the determination, either directly in expressed plant juice or in crude alkaloid extracts of plant material, of 5-hydroxytryptamine (serotonin), N,N-dimethyl-5-hydroxytryptamine (bufotenine), 5-methoxytryptamine, and N,N-dimethyl-5-methoxytryptamine in the presence of tryptophan, tryptamine, N,N-dimethyl-5-methyltryptamine, N,N-dimethyltryptamine, 3-(2-dimethylaminoethyl)-indole (gramine), *p*-(2-dimethylaminoethyl)-phenol (hordenine), or 3-indolylacetic acid.

MATERIALS AND METHODS

Preparation of samples

Fresh plant leaves were exposed to diethyl ether in a closed container for 3–5 min. Then the juice was expressed by the technique of MCCOMB AND RENDIG[6], and a quantity estimated to contain 10–100 μg of tryptamine derivatives was spotted on Whatman No. 1. chromatographic paper.

The tryptamines were isolated from dried leaf material by a procedure similar to that of KEFELI AND TURETSKAYA[7]. A weighed sample (40 mesh) was extracted with absolute methanol in a Soxhlet apparatus for approximately 7 h. The methanol was then evaporated, and 3–4 volumes of water were added to the liquid residue. After acidification with 0.1 *N* HCl, the mixture was extracted twice with an equal volume of toluene, then made alkaline with NH_4OH, and extracted three times with an equal volume of diethyl ether. The ether was evaporated and the residue made to volume with methanol. Samples estimated to contain 10–100 μg of tryptamine derivatives were spotted on the chromatographic paper.

Paper chromatography

Included on each chromatogram were known tryptamine compounds. These were dissolved either in absolute methanol or in acidified (HCl) aqueous solution for spotting.

The chromatogram was irrigated with butanol–acetic acid–water (12:3:5, v/v) for 16 h, dried and then dipped in fresh xanthydrol reagent (1 % xanthydrol and 10 % trichloroacetic acid in absolute methanol). After drying, the 5-O-substituted tryptamine derivatives appeared as blue areas on a pale pink background.

Colorimetric procedure

Those blue areas which had an R_F value corresponding to the known were cut out and placed in spectrophotometer tubes. Five milliliters of methanol were added and the stoppered tubes were inverted a few times. Then 0.5 ml of concentrated HCl was added and the tubes were shaken again. The pieces of chromatographic paper were removed, and the percent transmittance was read at 600 mμ. Areas of the paper similar in size and near the excised spots were treated in the same way and used to set the spectrophotometer at 100 % transmittance. The color was stable for at least 1 h. The amount of tryptamine in the sample was determined from a curve prepared from a chromatogram of the known compound (0–100 μg).

VARIABLES AND LIMITATIONS

The absorption spectra of the colors produced by the tryptamines and related compounds were determined, and the data for the compounds which produced xanthydrol-colored complexes are shown in Fig. 1. 5-Hydroxytryptamine, N,N-dimethyl-5-hydroxytryptamine, 5-methoxytryptamine, and N,N-dimethyl-5-methoxytryptamine produced curves almost identical in shape except for intensity. The

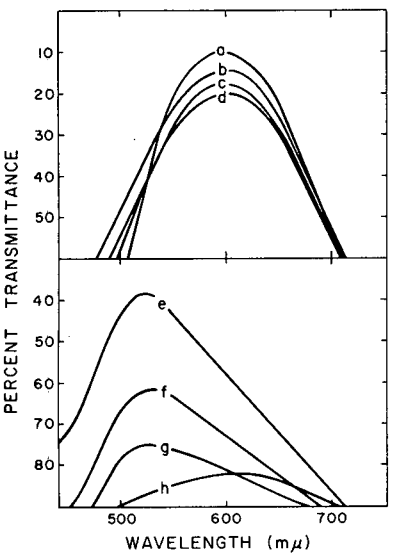

Fig. 1. Absorption maxima. a = 5-Hydroxytryptamine; b = N,N-dimethyl-5-hydroxytryptamine; c = 5-methoxytryptamine; d = N,N-dimethyl-5-methoxytryptamine; e = 3-indolylacetic acid; f = tryptamine; g = N,N-dimethyltryptamine; and h = N,N-dimethyl-5-methyltryptamine.

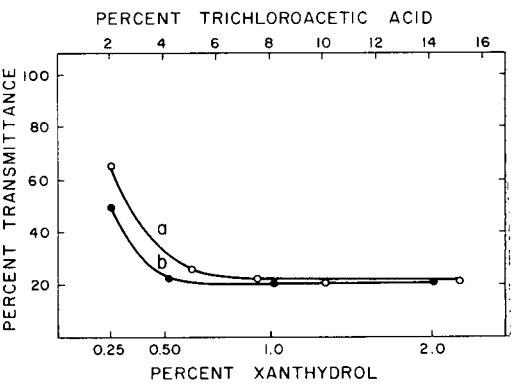

Fig. 2. Effect of reagent concentrations on transmittance. a = Trichloroacetic acid; b = xanthydrol.

absorption maximum in all instances occurred near 600 mμ. The absorption maximum for 3-indolylacetic acid, tryptamine, and N,N-dimethyltryptamine was near 525 mμ. N,N-Dimethyl-5-methyltryptamine failed to give a distinct maximum in the range of 500–700 mμ.

Initially a concentration of 15 % trichloroacetic acid, and xanthydrol concentrations of 0.25, 0.5, 1, and 2 % were tested. Maximum transmittance occurred with 1 and 2 % xanthydrol (Fig. 2).

Using 1 % xanthydrol 5, 7.5, 10, and 15 % trichloroacetic acid concentrations were tested. Maximum transmittance was produced with 10 % trichloroacetic acid (Fig. 2).

The xanthydrol reagent must be prepared directly before use, as maximum color development is not obtained when the reagent has been prepared longer than 2 h.

Plots of the log of the transmittance against the concentration over the range of 25–100 μg of the 5-O-substituted tryptamines gave straight lines (Fig. 3).

DISCUSSION

Of the eleven tryptamines and related compounds which might be expected to occur in plant material[1], only 5-hydroxytryptamine, N,N-dimethyl-5-hydroxytryptamine, 5-methoxytryptamine, and N,N-dimethyl-5-methoxytryptamine produced a xanthylium·HCl salt the color of which was proportional to the concentration of the indole at 600 mμ. Gramine, hordenine, and tryptophan did not form colored complexes. 3-Indolylacetic acid, tryptamine, and N,N-dimethyltryptamine produced similar purple colors, all of which had absorption peaks at 525 mμ. No distinct absorption maximum was found for the color produced by N,N-dimethyl-5-methyltryptamine.

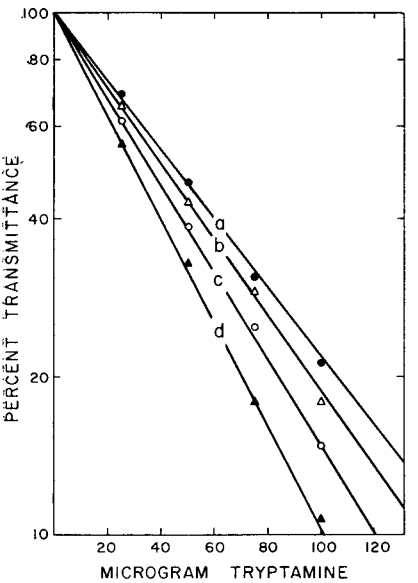

Fig. 3. Log of percent transmittance *vs.* μg tryptamine. a = N,N-Dimethyl-5-methoxytryptamine; b = 5-methoxytryptamine; c = N,N-dimethyl-5-hydroxytryptamine; and d = 5-hydroxytryptamine.

TABLE I

N,N-DIMETHYL-5-METHOXYTRYPTAMINE IN *Phalaris tuberosa* LEAVES

Leaf age (days)	*Leaf condition*	*Tryptamine concentration (% dry weight)*
7	fresh	0.236
9	fresh	0.105
21	fresh	0.077
21	frozen (3 days)	0.076
21	dried	0.071

Substitution of trichloroacetic acid for the acetic acid recommended by SCHREIER AND GAEDTKE[3] produced an intense color directly after the chromatogram was dipped and dried. Exposure to HCl fumes was not required. Because 9-substituted xanthylium salts are converted by alcohols to colorless ethers, it was necessary to add HCl to reform the colored indole-xanthylium·HCl complex. A more intense color was produced when the HCl was added after, and not with, the methanol. The color developed instantly. These results are in contrast to those obtained by the method of WILLIAMS[5], in which the color development time is critical.

The content of N,N-dimethyl-5-methoxytryptamine in leaves of *Phalaris tuberosa*, as determined by the proposed method, is shown in Table I.

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CHROM. 3865

THE MECHANISM OF ION EXCHANGE WITH ALGINIC ACID

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SUMMARY

The exchange mechanism which determines the retention capacity of alginic acid has been investigated. Chromatographic, pH, and viscosity measurements, performed with several metal ions, have permitted us to show that ion exchange is not the only mechanism but that the influence of the two vicinal hydroxyl groups on the retention capacity of alginic acid is also important.

INTRODUCTION

In previous papers[1,2] the characteristics of alginic acid as a new stationary phase for TLC and the chromatographic behavior of most elements on alginic acid thin layers were described. However, in these studies, the retaining mechanism of alginic acid for different metal ions was not investigated. Alginic acid is regarded by some workers[3,4] as a pure ion-exchanger which cross-links with carboxyl groups of different macromolecules, while SCHWEIGER[5,6] suggests that most divalent metal ions form complexes involving two carboxyl groups of one macromolecule and two vicinal hydroxyl groups of the same or another macromolecule.

Therefore we investigated the retaining mechanism of alginic acid to see whether ion-exchange is the essential parameter which determines the retention capacity of the alginic acid or whether the influence of other parameters such as the formation of chelate complexes and absorption, independent of the ionic groups of the polymer, is also important.

EXPERIMENTAL

Chromatographic studies

The chromatographic work on alginic acid was performed as described in a previous paper[1]. To prepare acetylated alginic acid thin layers, 6 g of the exchanger (D.A. = 1.7) were dispersed in 20 ml of a water–isopropanol mixture (1:1 by volume).

pH studies

Solutions. 0.1 *M* solutions, neutral or weakly acid of the following metal salts were prepared:

$Ba(NO_3)_2$, $Pb(NO_3)_2$, $Sr(NO_3)_2$, $Ca(NO_3)_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $BeSO_4 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $HgCl_2$, $AgNO_3$, $InCl_3$, $CrCl_3 \cdot 6H_2O$, $Ce(NO_3)_3 \cdot 6H_2O$, NaCl, LiCl, KCl and CsCl. Owing to the low solubility of Tl_2SO_4, an 0.05 *M* solution of this salt was employed.

The solutions were titrated according to customary analytical procedures.

Apparatus. pH measurements were performed with a Methrom Herisau Compensator E 388 pH-meter.

Method. 50 mg of alginic acid are dispersed in 10 ml of distilled water. The suspension is shaken for 1 h and the pH measured. 0.1 *M* solutions of the metal salts are added and the pH again determined after the samples have been shaken for at least 1 h.When acetylated alginic acid (D.A. = 1.7) was employed, 65 mg was weighed out in order to have the same concentration of carboxyl groups.

In the case of carboxymethylcellulose, 200 mg of the exchanger in the acid form are necessary because, as shown from curves (a) and (b) in Fig. 1, relative to 100 mg of alginic acid and 100 mg of carboxymethylcellulose, the exchange capacity of the latter is four times smaller than that of alginic acid.

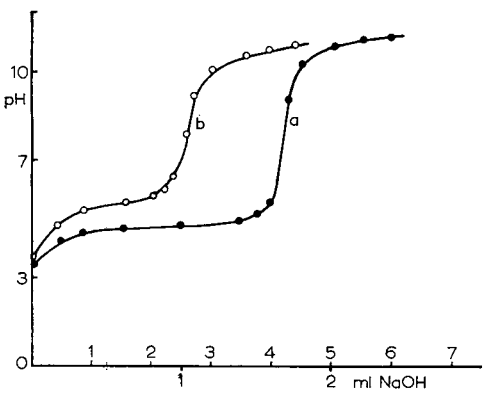

Fig. 1. Titration curves of alginic acid (a) and carboxymethylcellulose (b) with 0.1 *N* sodium hydroxide solution. The expanded scale is referred to carboxymethylcellulose.

Preparation of acetylated alginic acid

50 g of finely powdered sodium alginate are dispersed in 250 ml of 2 *M* HCl. The resulting product is separated, washed with distilled water to complete the removal of chloride ions and activated by washing twice with 250 ml of glacial acetic acid to reduce the water content to a small amount.

Activated alginic acid, glacial acetic acid (300 ml) and acetic anhydride (300 ml) are mixed in a Hobart mixer for 15 min. While mixing, 2 ml of perchloric acid (70 %) are added at such a rate that the temperature does not exceed 40°. This requires about 2 h. After a total reaction time of about 3 h, the mixture is filtered, washed with isopropyl alcohol and dried at room temperature. The yield of partially acetylated alginic acid depends on the reaction time.

Acetyl determination and preparation of ammonium acetyl alginate

The acetyl determination and the preparation of ammonium acetyl alginate

were performed according to SCHWEIGER[7]. Products with a D.A. of 0.93 and 1.7 were obtained.

RESULTS AND DISCUSSION

Comparison between alginic acid and acetylated alginic acid

If alginic acid behaves as a chelating agent, its retention capacity with respect to structurally similar ion-exchangers should be greater. So alginic acid has been compared with the acetylated alginic acid (D.A. = 1.7) since the latter cannot form chelates with metal ions.

The acetylation method according to SCHWEIGER[7] is preferred since the degradation of alginic acid is reduced to a minimum.

From the R_F values, reported in Table I, it can be seen that the different ions are retained on alginic acid much more than on acetylated alginic acid. Moreover the differences between the R_F values on acetylated alginic acid are not so marked as on alginic acid, confirming that the selectivity of the latter is due to the hydroxyl groups.

Such behavior confirms the hypothesis of chelate formation by alginic acid with respect to several ions, although the influence of a steric hindrance (due to the acetyl groups) on the bond between the carboxyl groups and the metal cation cannot be excluded.

TABLE I

R_F OF SOME ELEMENTS ON ALGINIC ACID (AA) AND ACETYLATED ALGINIC ACID THIN LAYERS (AAA)
Eluent: 0.01 and 0.05 *M* HNO_3.

Ion	*AA*		*AAA (D.A. = 1.7)*	
	0.01 M HNO_3	*0.05 M HNO_3*	*0.01 M HNO_3*	*0.05 M HNO_3*
Ag(I)	0.07	0.26	0.39	0.80
Tl(I)	0.05	0.12	0.38	0.80
Cu(II)	0.00	0.16	0.12	0.68
Zn(II)	0.02	0.30	0.24	0.76
Ni(II)	0.03	0.30	0.25	0.77
Pb(II)	0.00	0.00	0.00	0.11
Mg(II)	0.08	0.42	0.30	0.9
Ba(II)	0.00	0.05	0.11	0.67
Ga(III)	0.00	0.06	0.00	0.41
In(III)	0.00	0.00	0.00	0.06

In this connection it would be interesting to know the number of carboxyl groups participating in the exchange reaction with the polyvalent cations in the case of alginic acid and acetylated alginic acid.

By applying the law of mass action to the exchange reaction: $x\mathrm{H_R} + \mathrm{M}^{x+} = \mathrm{M_R} + x\mathrm{H}^+$ we obtain, by simple considerations, the relation: $x\mathrm{pH} = R_M + \text{const.}$ (ref. 8).

It is therefore possible to determine the number of carboxyl groups concerned in the exchange reaction through the R_M values, relative to eluents with different mineral acid concentrations.

In Figs. 2 and 3 the R_M values of several cations on alginic acid and acetylated

alginic acid are plotted *vs.* the log[HNO_3] in the eluent. The R_M/pH ratio increases with increasing valency of the ion and a straight line is observed even if the straight plots can be obtained only in a particular pH range. This is the case for Ag(I) and, particularly, for Ba(II), Sr(II), Pb(II) and Tl(I). Such cations do not travel with the solvent front even for high concentration of mineral acids in the eluent and therefore reveal the presence of a sorption by the thin layer.

Sorption on cellulose ion exchangers has been reported recently by MUZZARELLI[9]. According to this author the sorption is due to the interactions of the cation with the hydroxyl groups of the macromolecule. The values reported in Table II of the slopes

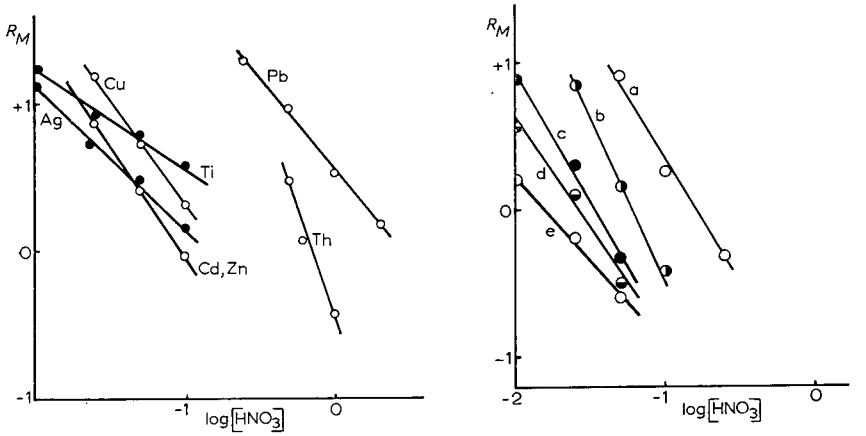

Fig. 2. R_M *vs.* log [HNO_3] in the eluent on alginic acid thin layers.

Fig. 3. R_M *vs.* log [HNO_3] in the eluent on acetylated alginic acid thin layers. (a) = Pb; (b) = Ga; (c) = Cu, Ba; (d) = Cd, Ni, Co, Zn, Ca, Sr; (e) = Ag, Tl.

TABLE II

SLOPES OF THE R_M–LOG [HNO_3] RELATIONSHIP FOR IONS OF VARIOUS IONIC CHARGES ON ALGINIC ACID (AA) AND ACETYLATED ALGINIC ACID (AAA) THIN LAYERS

Ion	*Charge*	*Slope*	
		AA	*AAA*
Ag	1	0.9	1.0
Tl	1	0.7	1.0
Pb	2	1.3	1.6
Ba	2	0.9	1.6
Sr	2	0.9	1.5
Cu	2	1.4	1.6
Cd	2	1.4	1.5
Ni	2	1.5	1.5
Co	2	1.5	1.5
Zn	2	1.5	1.5
Ca	2	1.3	1.5
Mg	2	1.4	1.4
Ga	3	2.4	2.2
In	3	2.4	n.d.
Th	4	3.0	n.d.

of the straight lines, relative to alginic acid and acetylated alginic acid, indicate a number of carboxyl groups equal to the valence of the ion. The difference between the experimental and theoretical values must be attributed, in addition to the sorption phenomena, to the formation of a pH gradient along the thin layer already observed in the case of alginic acid[1] and which is also present in the case of acetylated alginic acid.

pH measurements

On the basis of the above mentioned ion exchange reaction, when a metal salt solution is added to a suspension of alginic acid, the pH decreases and the magnitude of the decrease depends on the affinity between alginic acid and the metal ion.

We have thus decided to use the increase of the acidity, due to the ion exchange reaction, for the following purposes:

(1) to evaluate the affinity of different ions with respect to the acetylated and non-acetylated alginic acid;

(2) to see whether it is possible to correlate the data obtained in such a way with the chromatographic data;

(3) to understand the ion exchange mechanism by the comparison, under the same experimental conditions, of the data obtained on alginic acid with that on the acetylated alginic acid.

The investigation was also extended to carboxymethylcellulose, an ion exchanger widely employed in chromatography and structurally similar to the alginic acid.

The pH values, obtained after the addition of metal salt solutions to aqueous

TABLE III

pH OF AQUEOUS SUSPENSIONS OF ALGINIC ACID (AA) (INITIAL pH = 2.97), ACETYLATED ALGINIC ACID (AAA) WITH D.A. 0.93 (INITIAL pH = 3.32) AND 1.7 (INITIAL pH = 3.84) AND CAREOXYMETHYLCELLULOSE (CMC) (INITIAL pH = 3.21) AFTER THE ADDITION OF METAL SALT SOLUTIONS (MEQUIV. SALT/MEQUIV. EXCH = 1.43)

Metal salt	*AA*	*AAA (0.93)*	*AAA (1.7)*	*CMC*
$AgNO_3$	2.42	2.67	2.72	2.82
Tl_2SO_4	2.64	2.80	2.86	3.06
CsCl	2.66	2.80	2.82	2.94
KCl	2.72	2.89	—	—
NaCl	2.80	2.90	—	—
LiCl	2.90	2.90	2.91	3.06
$Pb(NO_3)_2$	2.04	2.24	2.53	2.20
$Ba(NO_3)_2$	2.20	2.48	2.77	2.56
$Sr(NO_3)_2$	2.34	2.52	2.78	2.67
$CuSO_4$	2.40	2.47	2.59	2.56
$Cd(NO_3)_2$	2.41	2.46	2.57	2.58
$Ca(NO_3)_2$	2.44	2.68	2.78	2.66
$BeSO_4$	2.60	2.74	2.76	2.88
$ZnSO_4$	2.61	2.76	2.78	2.85
$CoSO_4$	2.61	2.75	2.78	2.84
$NiSO_4$	2.62	2.76	2.78	2.84
$MnSO_4$	2.72	2.78	2.80	2.85
$MgSO_4$	2.74	2.88	2.96	2.88
$InCl_3$	1.98	2.18	2.23	2.28
$Ce(NO_3)_2$	2.12	2.36	2.46	2.54
$CrCl_3$	2.18	2.41	2.48	2.60

suspensions of samples of alginic acid, acetylated alginic acid with D.A. 0.93 and 1.7 and carboxymethylcellulose, are reported in Table III. From the above data it is possible to establish an affinity scale for the ions with respect to the ion exchangers employed (see below). Elements with pH values which differ $\pm$ 0.02 are considered to have the same affinity.

Alginic acid
(a) Ag > Tl, Cs > K > Na > Li.
(b) Pb > Ba > Sr > Cu, Cd > Ca > Be, Zn, Co, Ni > Mn, Mg.
(c) In > Ce > Cr.

Aceiylated alginic acid (*D.A.* = 0.93).
(a) Ag > Cs, Tl > K, Na, Li.
(b) Pb > Cu, Cd, Ba > Sr > Ca > Be, Zn, Co, Ni, Mn > Mg.
(c) In > Ce > Cr.

Acetylated alginic acid (*D.A.* = 1.7)
(a) Ag > Cs > Tl, Li.
(b) Pb > Cu, Cd > Ba, Sr, Ca, Be, Zn, Co, Ni, Mn > Mg.
(c) In > Ce, Cr.

Carboxymethylcellulose
(a) Ag > Cs > Tl, Li.
(b) Pb > Cu, Cd, Ba > Ca, Sr > Zn, Co, Ni, Mn > Be, Mg.
(c) In > Ce > Cr.

In the case of alginic acid we can draw the conclusion that, for ions of the same group of the periodic table, the affinity for alginic acid correlates with the size of the hydrated ionic radius and decreases in the following sequence:

$Cs^+ > K^+ > Na^+ > Li^+$; $Ba^{++} > Sr^{++} > Ca^{++} > Mg^{++}$; $Cd^{++} > Zn^{++}$

These results agree with those for carboxy cellulose[10]. The affinity scale for acetylated alginic acid and carboxymethylcellulose is different from that for alginic acid. On increasing the degree of acetylation the affinity scale changes and the selectivity of alginic acid with respect to several ions decreases. Such results are in agreement with the chromatographic ones.

Characteristics of metal alginates

The data reported have shown that the retention capacity of alginic acid depends, in addition to ion exchange, on other parameters among which the type of chelate involved seems to be important.

Therefore we tried to correlate the data in Table III with that given in Table IV regarding the characteristics of alginates with a variable degree of acetylation.

Univalent ions

The univalent ions do not cross-link as the viscosity measurements show. Using a Hoeppler viscosimeter, we observed no increase in the viscosity of sodium

TABLE IV

PRECIPITATION REACTIONS WITH METAL IONS[a]

Metal ion	*D.A.*		
	0	*0.93*	*1.7*
Ag(I)	+	+	—
Tl(I)	+	—	—
Cs(I)	—	—	—
Pb(II)	++	++	++
Cu(II)	++	++	++
Cd(II)	++	++	++
Ba(II)	++	++	—
Sr(II)	++	++	—
Ca(II)	++	++	—
Zn(II)	++	++	—
Co(II)	++	+	—
Ni(II)	++	+	—
Be(II)	+	+	—
Mn(II)	+	—	—
Mg(II)	—	—	—
In(III)	++	++	++
Ce(III)	++	++	++
Cr(III)	++	++	++
Th(IV)	++	++	++

[a] ++ = gelatinous precipitate; + = grainy gel; — = no reaction.

alginate solutions resulting from the addition of a thallous sulphate solution. However, unlike the alkali ions, Ag(I) and Tl(I) form insoluble alginates in some cases. Ag(I) forms a precipitate with sodium alginate and ammonium acetyl alginate with D.A. 0.93 but not with the one with D.A. 1.7, although it shows a similar affinity in both cases from the pH values reported in Table III. This behavior indicates that the presence of intramolecular chelates is still possible with alginic acid having D.A. 0.93.

Divalent ions

The divalent ions bind two carboxyl groups. For convenience such ions have been divided into three groups:

(a) Ba, Sr, Ca, Co, Ni, Zn.

(b) Pb, Cu, Cd.

(c) Mn, Mg, Be.

Group (a): The behavior of these ions is in agreement with the formation of intermolecular chelates as put forth by SCHWEIGER[6].

Group (b): The pH data and the type of precipitate obtained with these elements indicate the presence of cross-linkages between the macromolecules. Such results, which are in agreement with the measurements of viscosity performed by HAUG AND SMIDSROD[11], disagree with those of SCHWEIGER[6] for Pb(II). Furthermore, the chromatographic behavior of Cd(II), which is retained less with respect to the others, cannot be explained as it shows an affinity similar to that of Cu(II).

Group (c): For these elements the formation of cross-linkages is excluded. With respect to Be(II), however, the precipitates obtained with sodium alginate and ammonium acetyl alginate with D.A. 0.93 but not with D.A. 1.7, seem to indicate that

the formation of basic salts[6] is not the only criterion responsible for the precipitation but it is even possible that intramolecular non-cross-linked complexes are formed.

Tri- and tetravalent ions

In the pH range, at which the chromatographic measurements have been performed, tri and tetravalent ions seem to bind respectively three and four carboxyl groups.

Therefore it is reasonable to assume that carboxyl groups of different chains are participating in the reaction; this is in agreement with the formation of gelatinous precipitates even with high acetylated alginates.

Nevertheless we must consider, in the case of ions with strongly acidic characteristics such as Al(III), Fe(III) and Sn(IV), that the formation of the corresponding alginates may be affected by the presence of hydroxylated species of these ions and that, on increasing the pH, the number of carboxyl groups participating in the reaction may decrease. This behavior is confirmed by the formation of basic alginates and by the lack of a viscosity maximum in their suspensions[6].

CONCLUSION

The chromatographic, pH and viscosity data suggest the presence of mechanisms, different from ion exchange alone, which determine the retention capacity of alginic acid.

These effects are primarily due to the presence of two vicinal hydroxyl groups, and widely influence the tendency of the various ions to coordinate these groups. While for some ions (Ba, Sr, Ca, Zn, Co and Ni) a real chelation is probable, in other cases the great influence of the hydroxyl groups is still evident. In fact, it is sufficient to block the hydroxyl groups in order to decrease the selectivity of alginic acid and to obtain an ion exchanger that does not show substantial differences from exchangers with similar characteristics, such as carboxymethylcellulose. Thus it seems justified that alginic acid should be considered as a new stationary phase in TLC, particularly suitable for analytical purposes.

ACKNOWLEDGEMENT

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CHROM. 3885

ION-EXCHANGE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS ON ALGINIC ACID

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SUMMARY

Alginic acid was tested as an adsorbent in the ion-exchange chromatography of 31 amino acids on thin layers, the eluents being hydrochloric acid, acetic acid, water, potassium nitrate solutions and aqueous organic solvents. The behaviour of the amino acids on alginic acid was compared with their behaviour on carboxymethylcellulose. An ion-exchange mechanism operates in the case of basic amino acids. As regards the neutral amino acids, their affinity for alginic acid seems to be influenced by steric hindrance.

INTRODUCTION

Ion-exchange chromatography is simpler to perform and gives more reproducible results than partition chromatography, and it is particularly suitable for the analytical separation of organic ions with similar acidic or basic properties. Those ion-exchangers which have a high selectivity and therefore respond to small differences in these properties are of particular interest. Alginic acid is known[1–3] to show such a selectivity in the case of numerous inorganic ions, and has now been tested in the field of organic ions. The present work was limited to amino acids, which had been studied before on ion-exchange papers[4], and are suitable for testing the selectivity of ion-exchangers.

EXPERIMENTAL

Unlike pure alginic acid, plates coated with a 4:1 (w/w) mixture of alginic acid and cellulose retain their ion-exchange capacity practically unchanged; they also ensure a better reproducibility than pure alginic acid plates. In order to coat four 20 × 20 cm plates, 6 g of alginic acid and 1.5 g of cellulose were suspended in 40 ml of water, and the suspension was applied (thickness 300 μ) with the aid of an automatic Chemetron applicator. Other plates were prepared with a suspension of 5 g of carboxymethylcellulose (Na^+ form) in 50 ml of water, and with a suspension of 4.5 g carboxymethylcellulose (H^+ form) in 40 ml of water. The plates were dried overnight at 18–22° prior to use.

Each amino acid was dissolved separately to form a 1% solution in 10% iso-

propanol. Some amino acids, such as tyrosine and cystine, required 0.1 *N* HCl as solvent. The amount of each sample (*cf.* Table I) was decided on the basis of the size of its spot and sensitivity of the visualizing agent, found in preliminary experiments. The samples were applied to the plates at the start line, 1.5 cm from the lower edge. The plates were then developed to a height of 11 cm, in about 30–35 min, by the ascending technique with hydrochloric acid, acetic acid, water and potassium nitrate solutions. With aqueous isopropanol the developing time was about 50–60 min. The visualizing reagent described by MOFFAT AND LYTLE[5] proved particularly suitable.

TABLE I

R_F VALUES OF AMINO ACIDS ON ALGINIC ACID THIN LAYERS

Amino acids	*HCl*		CH_3COOH	*Amount*
	0.01 mole/l	*0.05 mole/l*	(*1 mole/l*)	(*μg*)
Arg	0.02	0.10	0.00	2.0
Lys	0.02	0.14	0.02	2.0
Orn	0.03	0.14	0.02	2.0
His	0.03	0.12	0.02	2.7
$(Cys)_2$	0.06	0.16	0.07	1.7
Gly (NH_2)	0.12	0.39	0.09	1.5
Try	0.14	0.28	0.13	2.5
γ-AnB	0.14	0.50	0.10	3.0
β-AiB	0.16	0.54	0.11	3.5
β-Ala	0.16	0.50	0.10	1.5
Gly (OCH_2CH_3)	0.18	0.50	0.13	2.0
Cit	0.22	0.43	0.19	2.0
Dopa	0.22	0.46	0.22	1.3
Tyr	0.23	0.54	0.23	0.8
Gly	0.23	0.49	0.19	1.5
Glu	0.28	0.58	0.27	0.4
Glu (NH_2)	0.28	0.54	0.27	0.5
Phe	0.30	0.58	0.27	1.3
Ser	0.30	0.59	0.27	1.2
Met	0.31	0.58	0.28	1.2
Ala	0.33	0.61	0.26	1.3
α-AnB	0.34	0.65	0.29	1.0
Thr	0.35	0.66	0.30	1.7
Sar	0.35	0.62	0.32	3.0
Asp	0.35	0.60	0.33	2.0
Val	0.36	0.68	0.29	0.9
α-AiB	0.36	0.72	0.32	3.5
Ile	0.36	0.69	0.35	1.2
Pro	0.40	0.66	0.40	3.0
β-Cl-Ala	0.47	0.64	0.44	2.3
Tau	0.96	0.97	0.86	5.0

RESULTS

Acidic eluents

The data in Table I, obtained with HCl as eluent, show that cystine and the basic amino acids were well separated from the others, but even the latter began to be separated as the concentration of HCl was increased. In particular, arginine behaved as could be expected from the most basic amino acid. The behaviour of the cystine is

due to the partial bivalent character of this amino acid. The separation of β-alanine from α-alanine and of some isomeric aminobutyric acids demonstrates the resolving power of alginic acid, as does the separation of glutamic from aspartic acid (all the more useful as other weak ion exchangers are incapable of it[4]). As expected from its marked acidity, taurine (Tau) migrated with the solvent front, unlike the other amino acids.

The replacement of HCl by HAc lowered the R_F values (which was expected) and changed the elution pattern, particularly in the case of some neutral amino acids (see Table I).

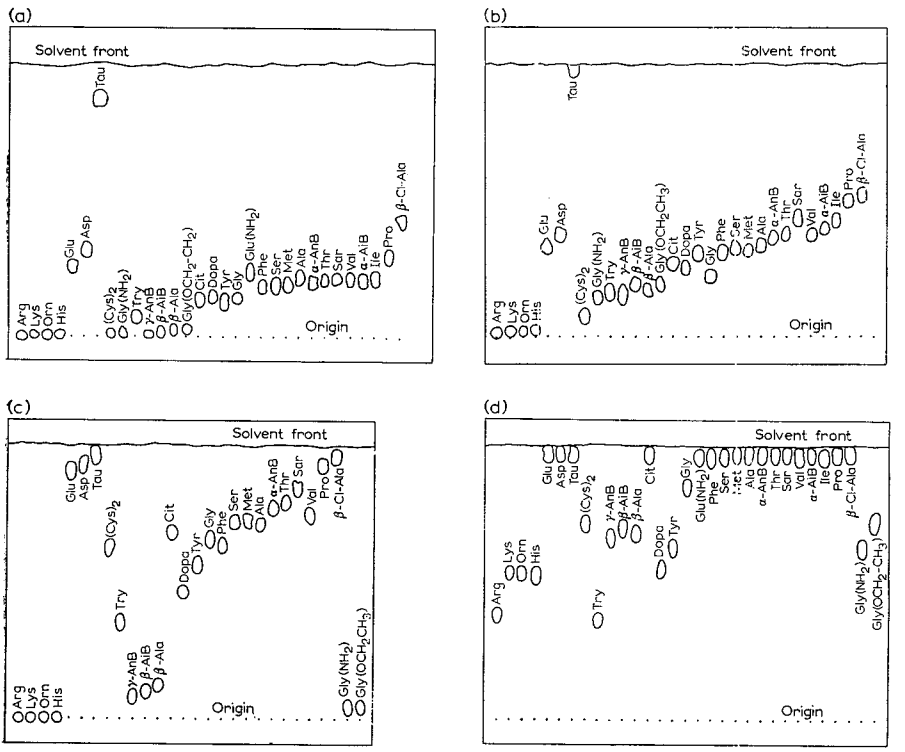

Fig. 1. Chromatograms of amino acids on alginic acid (a,b) and carboxymethylcellulose (c,d). Eluent: (a) and (c) H_2O; (b) and (d) 0.01 M KNO_3 solution.

Neutral eluents

Neutral salt solutions were tested as eluents to ascertain whether they were as efficient here as in the separation of some inorganic ions[6]. The use of water as eluent for the sake of comparison gave the chromatogram in Fig. 1a (alginic acid plate). This shows that basic amino acids, 4-aminobutyric acid (AnB), 3-aminoisobutyric acid (AiB), β-alanine and glycinamide did not migrate, an observation of analytical interest.

Neutral eluents were also useful in the comparison of alginic acid with carboxymethylcellulose, an ion exchanger of wide chromatographic use. This comparison helped to explain the behaviour of amino acids on alginic acids plates. The chromato-

grams in Fig. 1a–1d were obtained on alginic acid and on carboxymethylcellulose, with water and 0.01 *M* KNO_3 solution as eluents. The two adsorbents are seen to differ considerably in their ability to resolve the amino acids examined.

Mixed eluents

Aqueous organic solvents, recently used to elute organic[7] and inorganic[8,9] substances on ion exchangers, were found useful. This is particularly the case with aqueous isopropanol, which surpassed the reference eluent in resolving power on alginic acid thin layers. (*cf* R_F values in Table II and the chromatogram in Fig. 2). The selectivity increased and the R_F values decreased (except for some amino acids) when isopropanol was included in the eluent. The elution of amino acids with aqueous organic solvents is affected not only by ion exchange, but also by other factors such as liquid–liquid partition phenomena and a reduction in the polarity of the eluent in the exchanger[10].

TABLE II

R_F VALUES OF AMINO ACIDS ON ALGINIC ACID THIN LAYERS

Eluents: (1) 0.01 *M* HCl in 10% isopropanol; (2) 0.01 *M* HCl in 50% isopropanol; (3) 0.05 *M* HCl in 10% isopropanol; (4) 0.05 *M* HCl in 50% isopropanol.

Amino acids	*Eluent*			
	1	*2*	*3*	*4*
Arg	0.01	0.00	0.09	0.07
Lys	0.02	0.00	0.12	0.07
Orn	0.02	0.00	0.12	0.04
His	0.02	0.00	0.11	0.04
$(Cys)_2$	0.04	0.00	0.12	0.02
$Gly(NH_2)$	0.12	0.09	0.37	0.23
Try	0.14	0.33	0.32	e.s.[a]
γ-AnB	0.14	0.13	0.51	0.49
β-AiB	0.17	0.16	0.58	0.57
β-Ala	0.17	0.14	0.51	0.37
$Gly(OCH_2CH_3)$	0.19	0.18	0.56	0.48
Cit	0.20	0.16	0.43	0.39
Dopa	0.22	0.23	0.46	0.62
Tyr	0.23	0.29	0.54	0.74
Gly	0.19	0.09	0.49	0.37
Glu	0.28	0.20	0.58	0.58
$Glu(NH_2)$	0.28	0.16	0.58	0.57
Phe	0.30	0.36	0.58	0.74
Ser	0.30	0.15	0.59	0.50
Met	0.30	0.30	0.59	0.68
Ala	0.33	0.24	0.60	0.53
α-AnB	0.34	0.26	0.64	0.64
Thr	0.34	0.21	0.64	0.60
Sar	0.32	0.22	0.62	0.57
Asp	0.33	0.22	0.58	0.50
Val	0.36	0.35	0.68	e.s.
α-AiB	0.36	0.29	0.74	0.77
Ile	0.35	0.42	0.69	e.s.
Pro	0.37	0.31	0.62	0.56
β-Cl-Ala	0.46	0.22	0.63	0.47
Tau	0.85	0.54	0.90	0.56

[a] e.s. = Elongated spot.

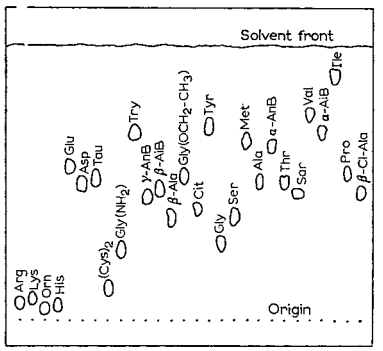

Fig. 2. Chromatogram of amino acids on alginic acid. Eluent: 0.05 M KNO_3 in 50 % isopropanol.

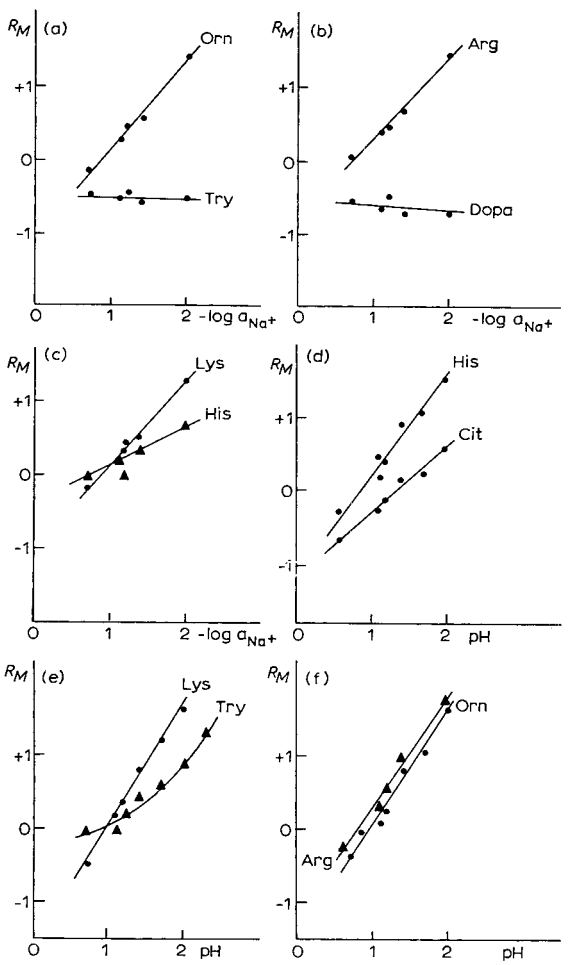

Fig. 3. R_M values *vs.* $-\log a_{Na^+}$ for some amino acids on sodium carboxymethylcellulose (a,b,c) and *vs.* pH on alginic acid thin layers (d,e,f). Eluents: NaCl and HCl solutions.

Retention mechanism

The good resolution of amino acids within a given group on alginic acid suggests that ion exchange is not the only factor affecting the retention. However, as regards the basic amino acids, non-ionic interactions found in cellulose-based ion exchangers[4] are secondary to ion exchange. The use of the expression $pH = R_M + \text{const.}$ (ref. 11) gave the curves in Fig. 3 (a–c refer to carboxymethylcellulose and d–f to alginic acid). In the case of carboxymethylcellulose (Na^+ form), the slope of the straight lines for basic amino acids (which is equal to 1, except for histidine) indicates a purely ion-exchange mechanism. The behaviour of histidine, for which the slope is 0.53, is explained by the fact that the pH of the measurements was close to the isoelectric point of histidine, and so the R form predominated over the R^+ form. In the case of alginic acid as adsorbent, the slope of the straight lines (1.4–1.6) indicates that basic amino acids behave as divalent ions in the pH region examined. The slope of citrulline (Cit) (0.87) suggests a single charged cation. The discrepancy between the theoretical and the experimental values of the slope is probably due to a pH gradient along the plate[1].

The curves for the neutral amino acids, tryptophan and 3,4-dihydroxyphenylalanine (Dopa) in Fig. 3 indicate the complete absence of ion exchange on carboxymethylcellulose (Na^+ form). The curve for tryptophan on alginic acid shows that adsorption on the latter is not determined by ion exchange alone, another factor may be steric hindrance, due to the side chains of the amino acids and the adjacent OH groups of alginic acid, which influences the reaction between the ionic groups of the amino acid and the adsorbent.

The following values, obtained for some representative amino acids with 0.05 *N* HCl, reveal considerable differences in the R_F, according

COOH | CH_2 | NH_2	COOH | $CH–NH_2$ | CH_3	COOH | $CH–NH_2$ | CH_2 | CH_3	COOH | $CH_3–C–NH_2$ | CH_3
$R_F = 0.49$	$R_F = 0.61$	$R_F = 0.65$	$R_F = 0.72$

to whether the NH_2 group is attached to a primary, a secondary, or a tertiary carbon atom. These differences are attributable to differences in the steric hindrance of the side chains. Provided that this observation is of general validity, it can be utilized to separate substances with similar acidities or basicities but different degrees of steric hindrance.

CONCLUSIONS

The present results have confirmed that alginic acid is a useful stationary phase for chromatography of organic and inorganic ions. Alginic acid exhibits a selectivity which makes it different from other weak ion exchangers and renders it suitable for analytical work. It is also better than carboxymethylcellulose in this respect.

ACKNOWLEDGEMENT

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CHROM. 3877

DETECTION AND ESTIMATION OF CHONDROITIN SULPHATE IN SUBMICROGRAM QUANTITIES AS A COMPLEX ON ACRYLAMIDE GEL

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SUMMARY

A complex between chondroitin sulphate and the histone fraction F1 was formed on acrylamide gel and separated from excess of histone by an electrophoretic method. The stability of the complex was demonstrated by staining reactions and also by radioactive determinations, for which the histone was highly labelled with [^{14}C]-dimethyl sulphate before formation of the complex. The complex was resistant to electrophoresis for long periods compared with the time required to separate the complex from free histone. The radioactivity was proportional to the 0.7th power of the amount of chondroitin sulphate on the gel. 10^{-8} g of chondroitin sulphate could be detected by the amount of radioactivity bound.

INTRODUCTION

Precipitation of a chondroitin sulphate with histone was observed by AGRELL AND HEBY[1] using a double diffusion technique in agarose. The binding of histone by agar gel prevents the migration of the protein in an electrical field. In the present experiments a sample of chondroitin sulphate was applied to acrylamide gel by electrophoresis. After a short time interval, the current was switched off and a sample of the histone fraction F1 was applied to the same gel with the voltage gradient reversed. The histone and chondroitin sulphate bands crossed in the gel. If histone was in excess, a histone–chondroitin complex was left at the crossing point and excess histone migrated off the gel. The complex was immobile and stable to electrophoresis for a period many times that required to resolve the complex from free histone. In order to quantitate the interaction, the histone F1 was methylated with [^{14}C]-dimethyl sulphate. The application of the interaction to the estimation of submicrogram quantities of chondroitin sulphate is discussed.

MATERIALS AND METHODS

Chondroitin sulphate

This was prepared as the barium salt from bovine nasal cartilage by the method of MALAWISTA AND SCHUBERT[2]. The sulphur content (measured by Dr. G. WEILER

and Dr. F. B. STRAUSS, Oxford) was 3.1 %. The material was dissolved in 40 % w/v sucrose to give a series of dilutions from 0.01 to 3.0 mg/ml. These were stored frozen at —8°. The histone fraction F1 was that used in a previous investigation[3]. The protein was methylated by EDLBACHER's[4] method as follows: 30 mg of fraction F1 which had been prepared by precipitation as the hydrochloride from aqueous acetone was dissolved in 1.4 ml water. 0.6 ml 0.1 *N* NaOH was added and also 0.1 mC [^{14}C]-dimethyl sulphate (22.7 mC/mmole from the Radiochemical Centre, Amersham) dissolved in 0.1 ml ethanol. The system was mixed well and left stoppered at room temperature (22°) for 2 h. One drop of aqueous 0.5 % w/v bromophenol blue solution was added and 2 drops of 6 *N* HCl to render the solution acid. The methylated histone was precipitated by addition of 10 ml acetone and washed by decantation with ten washes of 10 ml acetone. The acetone was drained off, the protein allowed to become air dry and redissolved in 10 ml 40 % w/v sucrose. The solution was divided into several parts and stored frozen at —20°. The methylation was almost quantitative as judged by the radioactivity of the protein solution (10^7 disintegrations/min/ml). It is believed that methylation occurs at the ε-amino group of lysine. Some previous experiments (with Dr. G. WILTSHIRE) had shown a considerable (80 %) reduction in N-amino after methylation of bovine plasma albumin with excess dimethyl sulphate under alkaline conditions.

Acrylamide gel electrophoresis

The electrophoresis was done in capillary tubes (50 × 1 mm) obtained by cutting disposable 100 μl micropipettes (Dispo, Scientific Products, Evanston, Ill., U.S.A.) crosswise in half. The tubes were not cleaned prior to use. The tubes were filled by capillary action with the gel system described by REISFELD *et al.*[5]; the acrylamide concentration being 28 % w/v in the stock solution and 7 % in the final gel. The tubes were sealed at the lower end by standing them in a plastic clay (Seal-Ease, Clay-Adams Inc., New York). The gels were water-layered in the usual way. Before setting the top of the gel was sharpened by withdrawing some of the boundary into a fine syringe needle attached to a micrometer syringe (Kontes Glass Co., Vineland, N.J., U.S.A.) by narrow flexible tubing.

The clay seal was removed by cutting off the end of the tube with an ampoule file. The tubes were supported in a conventional two-tier apparatus made from Perspex (Imperial Chemical Industries). The water seal in which the tubes were supported was provided by a sheet of 1/8 in. thick (3 mm) non-intercellular neoprene (Expanding Rubber Company, Croydon, Surrey), sandwiched between the bottom of the upper buffer compartment and another layer of Perspex. The sandwich was held together with nylon nuts and bolts (Nyloy Screws, London) and butyl rubber washers. The buffer compartments were filled with the buffer system of NAGAI *et al.*[6].

Application of the samples was by means of a calibrated microsyringe (Hamilton, Whittier, Calif., U.S.A.). This was supported in a frame constructed from a desk lamp (Terry, Redditch, Worcs.) with an aluminium bar, 7.5 × 1.5 × 1.5 cm, with a spring clip in place of the lamp reflector and socket. The chondroitin sulphate was applied to the top of the gel and migrated downwards towards the anode. When the histone was added to the top of the gel the field was reversed so that the bottom compartment contained the cathode and the chondroitin sulphate already on the gel began to migrate upwards to meet the labelled histone. In either case, the magnitude

of the voltage across the electrodes was 100 V. After electrophoresis the gels were extracted by breaking the tubes by hammering with the "sharp" end of a glass pestle. The gels were protected during this procedure by placing the tubes in a groove in a Perspex block from which they projected only slightly. The broken glass was washed off by brief immersion in running tap water.

Staining procedures

Protein was located on the gel with 1 % w/v amidoschwarz (G. T. Gurr, London) in 7 % w/v aqueous acetic acid. Chondroitin sulphate was located with 0.1 % Alcian blue (G. T. Gurr, London) in 0.6 *N* HCl. After staining the background was reduced by washing in 7 % acetic acid and 0.6 *N* HCl respectively.

Radioactive counting

The gels were counted in a Packard TriCarb Scintillation Counter with phosphor as described by LAURENCE AND BUTLER[7]. The position of the complex was determined by staining some of a series of electropherograms for protein. The staining and washing necessary to determine the position of a band did not affect the radioactivity subsequently obtained and these stained gels could be included in the radioactive sample. As the position of a band did not depend upon the amount of chondroitin sulphate used, it was only necessary to stain some of the more dense complexes. A thin slice of the gel was removed from the origin and discarded. Two consecutive 0.5 cm segments of the gel were then cut out, the first of which contained the complex, the second was used as a blank. Radioactivity was eluted into the counting vials by leaving the gel segments in 0.4 ml 25 % w/v tetraethylammonium hydroxide for 2 days before adding 10 ml of the phosphor.

Radiological precautions

It should perhaps be emphasized that the methylated histone is highly radioactive and strongly bound. Care must therefore be taken to avoid personal contact with this reagent, and spent buffer and the capillary tubes after the electrophoretic run.

RESULTS

The formation of a histone–chondroitin sulphate complex on acrylamide gel and its subsequent stability and immobility are illustrated in Fig. 1. In these experiments the chondroitin sulphate (3 μg in 1 μl of solution) was allowed to run for 5 min before adding the unlabelled histone solution (2 μl $\equiv$ 6 μg protein) in excess and reversing the electrical field. Fig. 1 shows the results obtained at various times after the start of the histone migration. After 15 min the excess histone has already passed the chondroitin sulphate. The complex that resists electrophoresis for at least 90 min may be stained with either protein or mucopolysaccharide stains.

The stability of the complex is also indicated by radioactivity measurements on the extracted gels. Table I gives data of the counts/min obtained in an experiment similar to that shown in Fig. 1. Table I also contains the results of allowing the chondroitin sulphate to run for 30 min before addition of methylated histone and field reversal. The counts/min obtained for the shorter time of pre-running were higher

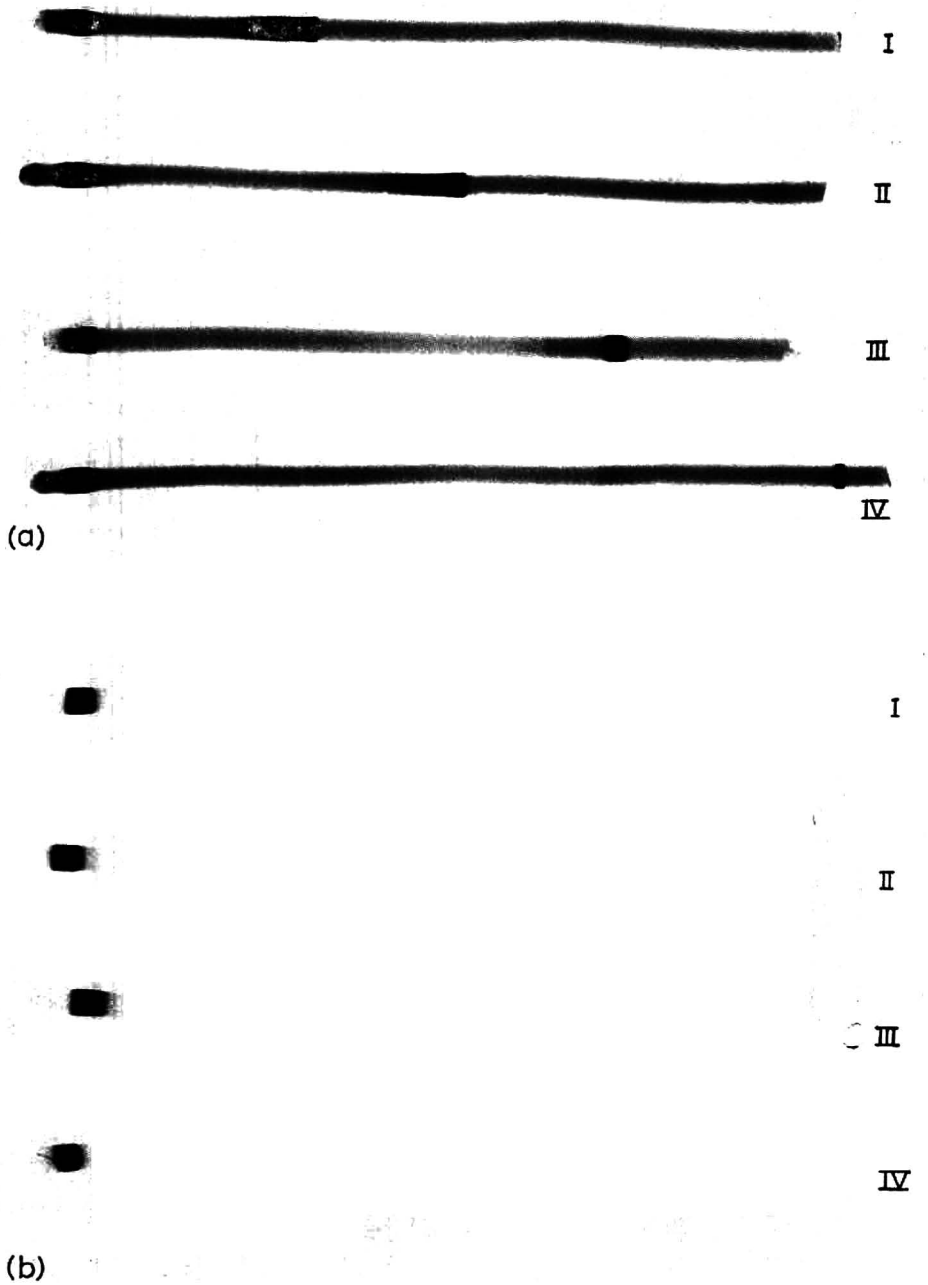

Fig. 1. Separation of excess histone and stability of the chondroitin sulphate–histone complex. (a) Protein staining; (b) alcian blue staining. Duration of the electrophoresis of histone: (i) 5 min, (ii) 30 min; (iii) 60 min; (iv) 90 min.

TABLE I

DETERMINATION OF THE STABILITY OF THE HISTONE–CHONDROITIN SULPHATE COMPLEX TO ELECTROPHORESIS

All table entries are in counts/min. 3 μg of chondroitin sulphate were allowed to react with an excess of histone. T_1 = time for which chondroitin sulphate was pre-run, T_2 = time of subsequent electrophoresis of labelled histone.

T_2 (*min*)	T_1 (*min*)	
	5	30
15	7250	6800
30	6600	5650
60	6950	5750
90	6200	5600
120	6100	5600

than those obtained for the longer time. When chondroitin sulphate is run for 30 min the complex is more diffuse, and it was necessary to take 2 cm pieces of gel in order to include the entire complex. After a fairly rapid fall in radioactivity within the first 30 min period, the values obtained between 30 min and 120 min showed a slower decrease which was very slight in the case of the 30-min pre-run.

The sensitivity of the complex formation was tested by taking a series of dilutions of chondroitin sulphate. 0.5 μl quantities of chondroitin sulphate were used and applied by means of a Hamilton 1 μl syringe with a Chaney adaptor and run for 5 min. 2 μl quantities of the stock methylated fraction F1 solution were then applied and the electrophoresis was continued for 90 min with reversed polarity. A blank sample of 0.5 μl 40 % sucrose was run and the radioactivity of this blank was subtracted from all the values of radioactivity obtained with the samples to correct for histone adsorbed to the gel. The results are shown in Fig. 2, and the relationship between net counts/min (measured over 10 min corrected for blank) and amount of chondroitin sulphate applied was linear when plotted on a logarithmic scale for both axes. The slope of the graph is 0.7 and so the counts/min were proportional to the

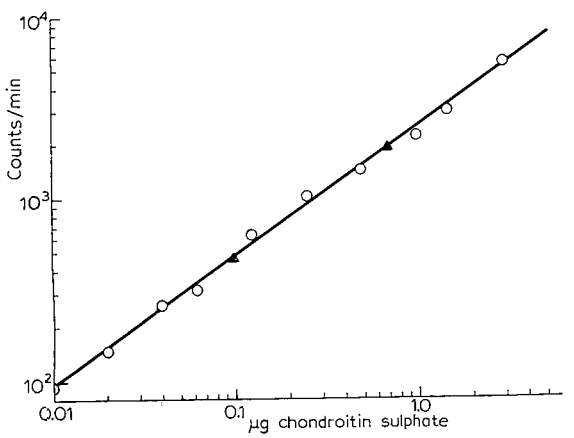

Fig. 2. Relationship between net counts/min extracted from the gel and the amount of chondroitin sulphate applied. O = known dilutions of chondroitin sulphate; ▲ = unknown dilutions with concentration subsequently revealed.

0.7th power of the chondroitin sulphate concentration under these conditions. In addition to the known dilutions, two samples were made up with concentration unknown to us until after the determination had been made. These samples are indicated on Fig. 2 by the point indicated by ▲. The lowest amount of chondroitin sulphate used (10^{-8} g) gave a net radioactivity (95.7 counts/min) that was still in excess of the blank value (85.1 counts/min) and so the gross radioactivity of this sample was more than twice "background". All samples were run in pentuplicate to allow for technical failures. Usually at least four samples survived to be counted.

DISCUSSION

The difference in the binding of methylated histone fraction F1 to a blank gel and to a similar gel containing 10^{-8} g chondroitin sulphate emphasises the relatively weak adsorption of the modified protein by the gel. The weight ratio of acrylamide to chondroitin sulphate was about 6×10^4 but the ratio of binding was about unity. Some of the "binding" of the histone to the blank gel may have been due to formation of aggregates that move only slowly in the gel. It was found that a considerable sticking (about 500 counts/min) of the radioactivity occurred to the first millimetre of the gel. This was removed in the experiments with radioactivity by rejecting a thin layer at the top of the gel. The radioactivity at the origin increased slightly over the range of chondroitin sulphate concentrations which suggests that a small part of the mucopolysaccharide sample did not penetrate the gel.

The histone fraction F1 was a cationic polymer readily available to us. This substance has the advantages of a relatively low and controlled molecular weight and the tendency to aggregate is not too pronounced[8]. From the work of AGRELL AND HEBY[1] it is obvious that other cationic polymers and perhaps some polyamines might be tried as a substitute for fraction F1. Methylation is a convenient method of labelling as the basic properties of the protein are conserved or perhaps even enhanced.

The reaction obtained is specific to acidic polysaccharides and other polymers that form aggregates with histone at the pH of the gel (4.6). It is necessary that the polymer should readily penetrate the gel; hyaluronic acid will not penetrate but heparin will do so. The latter forms a complex with similar properties to those described here. The binding for a given weight of heparin was about twice that for the same weight of chondroitin sulphate and this ratio corresponds roughly to the relative amounts of sulphate in the two substances. It is obviously necessary to liberate the chondroitin sulphate from its protein complex by hydrolysis of the protein. When this is done, competition between the histone and proteins in the sample for sites on the chondroitin sulphate should be minimized.

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Notes

CHROM. 3906

Low pressure gas chromatography

Experimental

A Beckman Model GC-2 gas chromatograph was modified so that in addition to the normal mode of operation the instrument could be operated with the carrier gas at the inlet of the column being at atmospheric pressure, and the exit at the detector being held at a reduced pressure. A diagram of the instrument with the modifications is shown in Fig. 1.

The modification of the Beckman GC-2 consisted of placing two three-way Hoke valves in the normal flow pattern of the instrument. One of the three-way valves was placed in the instrument so that the carrier gas to the column could be supplied from either of two different sources. The other three-way valve was placed on the exit end of the detector so that the detector could be vented to the atmosphere during normal operation or connected to a vacuum source during low pressure gas chromatography.

During low pressure chromatography the carrier gas to the chromatograph is supplied from a helium reservoir which is a stainless steel cylinder in which helium is held at atmospheric pressure. Helium from a Linde 1A size cylinder is continuously supplied at the bottom of the reservoir and allowed to exit at the top of the reservoir by either of two outlets. One exit is vented to the atmosphere and the other is connected to the three-way valve at the inlet of the chromatographic column. The flow of helium to the reservoir is controlled by a needle valve in the line from the helium cylinder to the reservoir. The flow rate of carrier gas through the column during low pressure chromatography is determined by measuring (with a soap film flow meter) the flow rates of helium out of the reservoir at the atmospheric vent when the connection to the chromatograph is closed and opened.

The vacuum source used during low pressure chromatography is a Gast vacuum pump Model 0211-V201-G10X equipped with a vacuum gauge. The vacuum pump is connected to the top of a vacuum reservoir which is a stainless steel cylinder of approximately one liter capacity. The vacuum reservoir is used to minimize any fluctuations in vacuum due to the mechanical action of the vacuum pump. The exit of the detector of the chromatograph is connected to the vacuum reservoir with the three-way valve. A trap was placed in the line connecting the chromatograph and the vacuum reservoir to collect the compounds eluted from the column before they went into the vacuum pump.

When the two three-way valves are in the position shown in Fig. 1, the gas chromatograph can be operated in the normal mode with the flow rate of the carrier gas through the column being determined by the pressure that is applied to the inlet of the column. By rotation of the two three-way valves, the chromatograph is operated in the low pressure mode with the flow rate of the carrier gas through the column

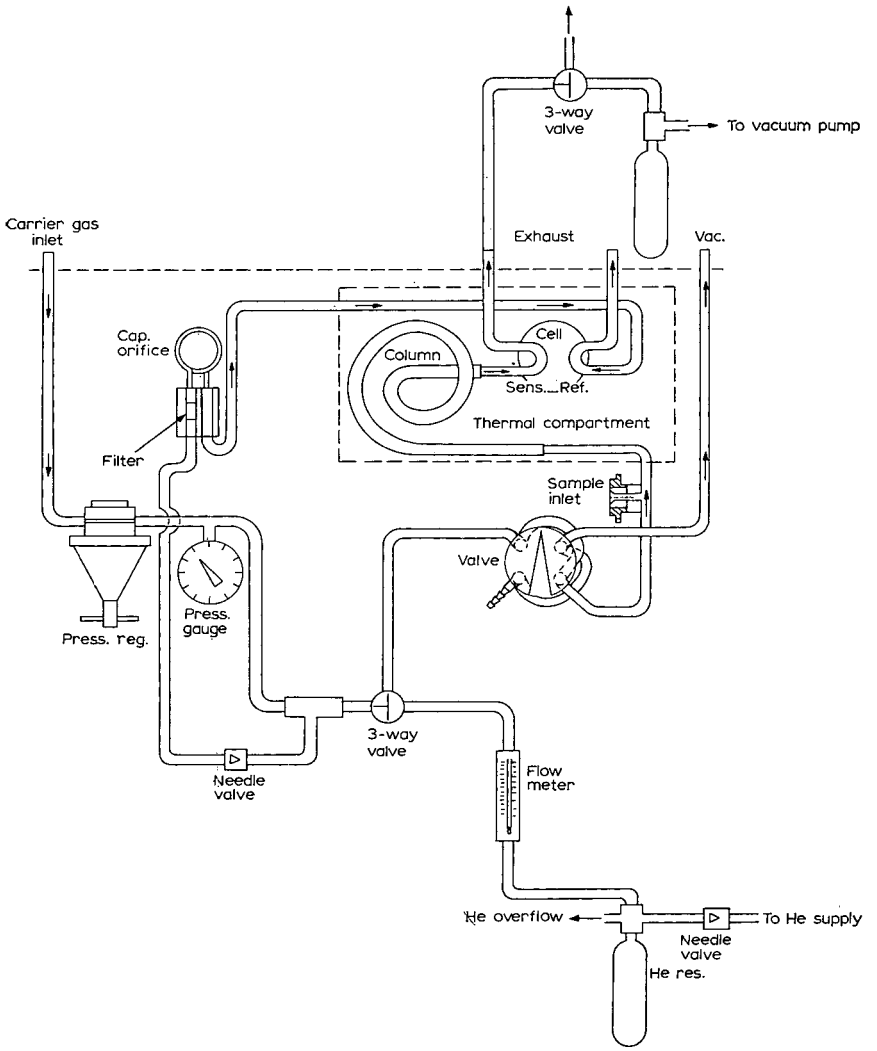

Fig. 1. A diagram of the modified gas chromatograph.

being determined by the amount of vacuum that is applied at the exit of the detector. The data collected were obtained with a vacuum of 26.5 in. of mercury at the pump.

The column used was stainless steel, 6 ft. by 2 mm I.D., packed with 10% SE-30 coated on 60–70 mesh HMDS treated Chromosorb W. The column temperature was held isothermal at 100° or 140° with a carrier gas flow rate of 24 ml per min for the normal mode and 20.3 ml per min for the low pressure mode. The detector current was 250 mA and the detector temperature was the same as the column, while the injection port was maintained at 208° for all samples. The sample size was 1 μl for all compounds tested. The recorder used was a Hewlett Packard Autograf with Model 7101B input module. The input was 1 mV and the chart speed was 0.5 in. per min.

Results and discussion

The results presented in Table I show that the retention time of the compounds chromatographed under the low pressure mode was approximately half of what it was under the normal mode.

The gas chromatograms of the compounds obtained under normal and low pressure modes are shown in Fig. 2. It may be seen from this figure that the chromato-

TABLE I

RETENTION TIMES OF SOME COMPOUNDS RUN UNDER NORMAL AND LOW PRESSURE MODES

Compound	*Column temperature (°C)*	*Retention time*	
		Normal mode	*Low pressure mode*
Formic acid	100	0.52	0.38
Water	100	0.62	0.40
Acetic acid	100	0.90	0.42
2,2,4-Trimethyl pentane	100	1.00	0.58
Propionic acid	100	1.78	0.92
2-Octanol	100	5.80	3.18
Phenyl ethanol	140	4.38	2.19
n-Dodecane	140	6.82	3.81
Phenyl ethyl acetate	140	8.60	4.60

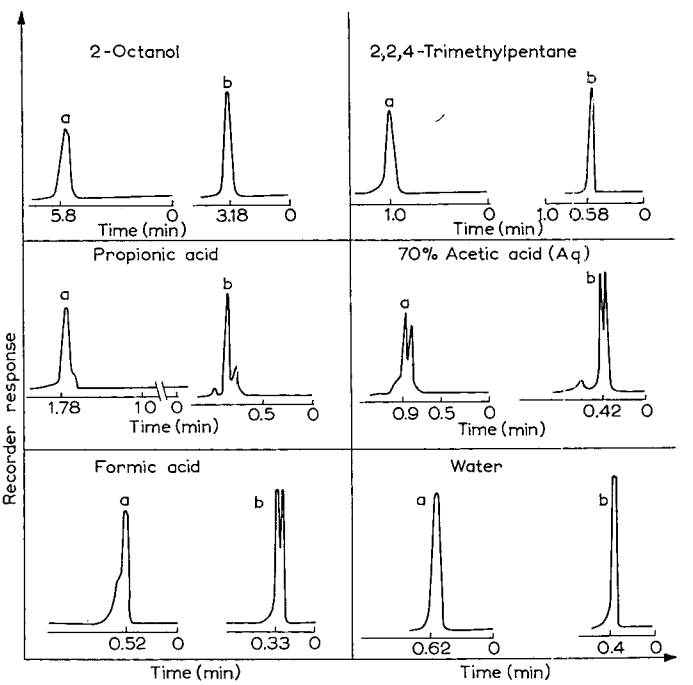

Fig. 2. Gas chromatogram of some compounds run under (a) normal mode, and (b) low pressure mode.

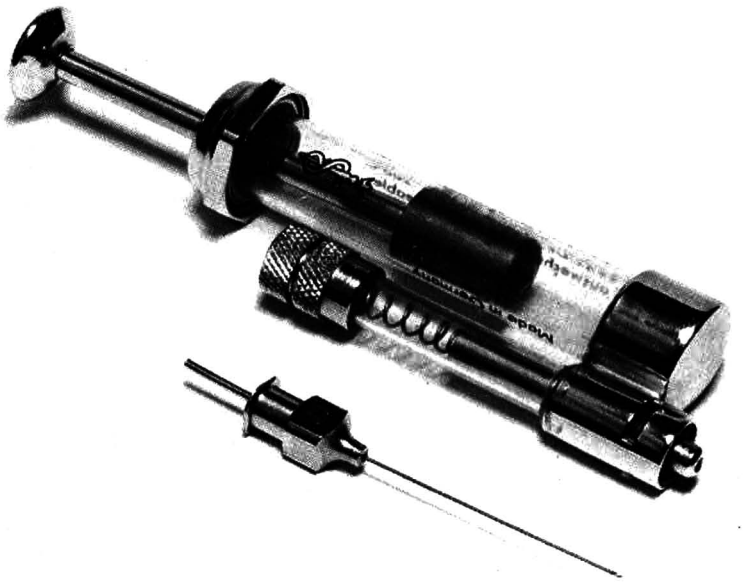

Fig. 1. Sampling device.

activated. The spring (7) is depressed only when the pressure exceeds that of the carrier gas, thus introducing the sample into the injector. The injection pressure remains constant throughout the operation.

Considerable care must be exercised in filling the cannula during quantitative experiments. The following procedure is recommended: place a transparent tube of plastic or silicone rubber over the rear end of the cannula and, through it, draw up the test mixture to a level of 1 cm. Attach a piece of tubing approximately 1 cm long to the front (pointed) end of the cannula and blow the sample through the cannula from back to front so that the surplus mixture is equally distributed between the tubes at the two ends of the cannula. Remove the tubing from the wider end, wipe the upper section of the cannula clean with a small piece of filter paper and then press the paper over the opening to draw off part of the test mixture from the front end of the cannula. Attach the cannula to the air-filled syringe. The tubing at the front end is removed immediately before injection into the gas chromatograph. The entire procedure outlined above takes approximately 30 sec. The cannulas are cleaned in the same way as pipettes (by blowing through water, solvent or air).

The degree of reproducibility was tested using cannulas with capacities of 2 μl and 0.5 μl. In the first series of experiments (2 μl) twelve injections were carried out with four different alcoholic solutions (Merck). The results are shown in Table I.

Table II shows the results obtained in the test series using the 0.5 μl cannula. For experiment (a) we employed a $1^0/_{00}$ alcoholic solution with a recorder attenuation of 32 × which produced a relatively low peak, while for experiment (b) we used

grams obtained under low pressure mode show sharper peaks than those obtained under normal mode. In the case of formic and propionic acids, the impurities which appeared as shoulders during the normal mode, gave sharp and well resolved peaks during the low pressure mode.

The method appears to hold out several advantages over the normal gas chromatographic analysis. Some of these would be the study of high boiling or low volatile compounds, and analysis of heat-labile compounds in addition to the faster elution and better resolution. A detailed work covering the study of these factors and others has been undertaken, and the results will be reported in subsequent communications.

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CHROM. 3904

A new sampler for gas chromatographic analysis of liquids and solutions

According to LEIPNITZ AND STRUPPE[1], one of the most important prerequisites for exact analysis is that the amount of injected material and the method of introduction be as precisely reproducible as possible. The samplers presently available fulfil this condition only partially and also present cleaning difficulties, especially when viscous substances are used. Nor do they have a constant injection pressure, except when used with the "Reprojector"*. This reprojector, however, is even more difficult to clean than the other samplers. Thus we have tried to develop an improved sampler which eliminates these deficiencies and at the same time permits a high degree of reproducibility.

Fig. 1 shows the newly developed sampler** which consists of two parts: a syringe, which does not come into contact with the test mixture, and a calibrated component, namely an interchangeable cannula made of stainless steel. The syringe is a standard 2 ml syringe modified by the addition of a pressure relief valve. The method of operation is illustrated in Fig. 2. The 2 mm^3 cannula containing the test sample (5) is attached to the cone of the air-filled syringe. Automatically the rear, wider end of the cannula (11) depresses the cylinder (8)—made airtight by a gasket (12)—of the pressure relief valve, thus effectively sealing the cannula. Next, the needle is inserted into the injector of the gas chromatograph and the plunger (2)

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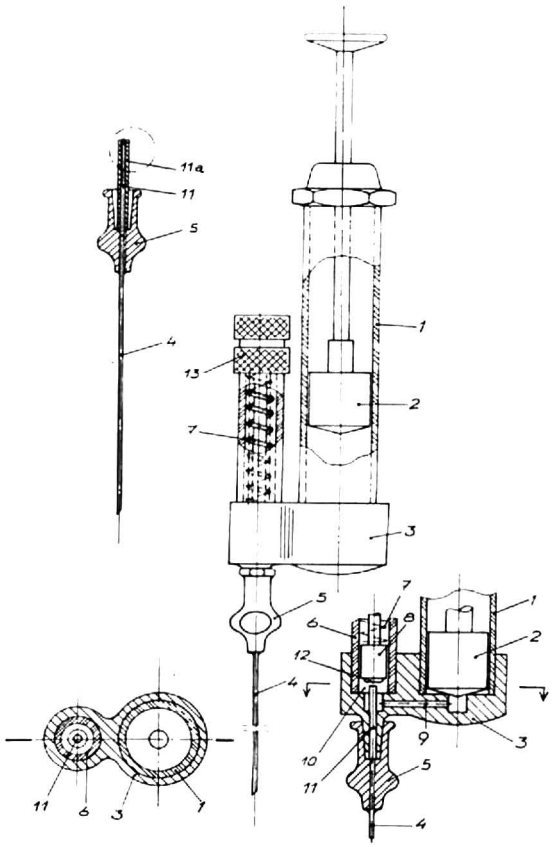

Fig. 2. Plan of sampling device.

TABLE I

DEGREE OF REPRODUCIBILITY USING CANNULAS WITH CAPACITIES OF 2 AND 0.5 μl AND DIFFERENT ALCOHOLIC SOLUTIONS

	(*a*) 0.8‰		(*b*) 1.6‰		(*c*) 2.8‰		(*d*) 3.0‰	
Peaks	63.2	64.2	65.0	64.5	113.1	113.9	120.5	121.0
	62.4	62.9	65.1	64.8	114.4	113.9	120.8	118.7
	64.0	63.5	64.6	64.6	113.2	114.8	120.1	119.9
	63.0	63.8	64.7	65.8	113.9	112.8	119.2	120.8
	64.2	63.2	63.5	63.8	113.2	112.8	120.0	120.6
	63.4	64.1	65.2	64.9	114.0	113.8	121.2	120.2
Mean values	63.5		64.7		113.6		120.2	
Deviation (%)	0.91		0.94		0.56		0.60	

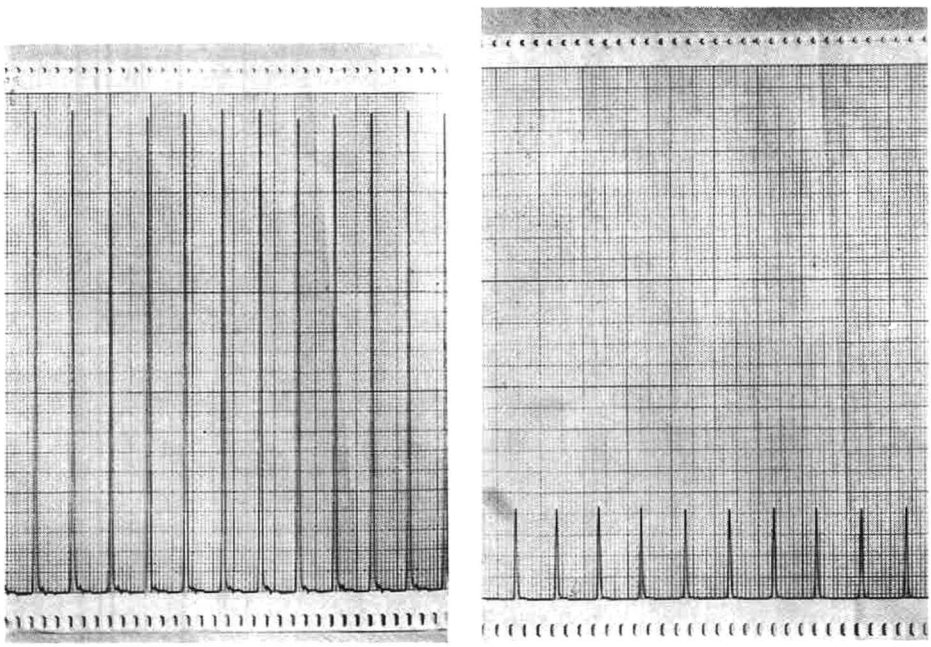

Fig. 3. (a) Results using 2 μl of a 3.0‰ solution of ethyl alcohol in water (Table Id); (b) results using 0.5 μl of a 1.0‰ solution of ethyl alcohol in water (Table IIa).

TABLE II

DEGREE OF REPRODUCIBILITY USING A CANNULA WITH A CAPACITY OF 0.5 μl AND DIFFERENT SOLUTIONS

	(*a*) 1.0‰ *alcoholic solution*[a]		(*b*) *Benzol*[b]		
Peaks	21.3	20.9	75.9	76.3	74.3
	21.2	21.2	74.7	75.3	74.7
	21.7	21.2	75.2	75.0	75.5
	21.5	21.4	76.0	75.2	74.6
	20.9		76.5	74.9	75.3
	20.9		76.0	75.6	74.9
Mean values		21.3		75.3	
Deviation (%)		1.17		0.82	

[a] Recorder attenuation 32 ×
[b] Recorder attenuation 3200 ×.

benzol with a recorder attenuation of 3200 ×. The peaks were measured in all experiments.

The following conditions prevailed: Varian aerograph 1525; detector, F.I.D. 150°; injector, 125°; columns, Hellcomid 100/120 mesh, Chromosorb white/hexamethyldisilicane 78°; carrier gas, N_2, 30 cc/min.

The mean error was calculated according to the usual formula

$$\xi = \sqrt{\frac{\Sigma(Mv-M)^2}{n-1}}$$

with M representing the measured value, Mv the mean value ($Mv = \Sigma M/n$, and n the number of experiments performed. The tables demonstrate that the degree of reproducibility achieved was sufficiently high to meet the standards demanded in exacting analytical work despite the fact that neither an internal standard nor an integrator was used.

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CHROM. 3914

The analysis of oils and fats by gas chromatography

VII. Separation of long-chain fatty alcohols as their trifluoroacetyl and trimethylsilyl derivatives

Long-chain fatty alcohols may be separated by gas chromatography but because of length of analysis time and the tendency for these substances to give tailing peaks it is usual to convert the alcohols to suitable derivatives before gas chromatography. Recently VANDENHEUVEL, GARDNER AND HORNING[1] evaluated ten derivatives for the gas chromatographic analysis of alcohols but only the C_{12}, C_{14}, and C_{16} saturated alcohols were analysed. WOOD[2] has shown that the trifluoroacetyl (TFA) and trimethylsilyl (TMS) derivatives were suitable for the analysis of alcohols. These derivatives have shorter retention times than the corresponding acetyl derivatives. He also indicated that the TFA derivatives of C_{18} unsaturated alcohols gave a better separation than the TMS derivatives on a polar stationary phase but no retention data were given.

ACKMAN[3] has suggested that it is probable that derivatives of unsaturated acids produced by modifying the carboxyl group will have the same Type II separation factors, and JAMIESON AND REID[4] found that long-chain methyl esters, alcohols, acetates and hydrocarbons had the same Type II separation factors on a BDS packed column. The present work investigates the separation of TFA and TMS derivatives of unsaturated alcohols on a polar and two low-polarity stationary phases and compares the separation factors obtained with those given by the corresponding acetyl derivatives.

Experimental

Alcohols were prepared from the corresponding methyl esters by lithium aluminium hydride reduction using the procedure previously described[4]. TFA and TMS derivatives were prepared from the alcohols by the methods described by WOOD and his coworkers[5–7].

Gas chromatography was carried out on a PE 800 gas chromatograph with nitrogen as the carrier gas and the following columns:

(1) EGSS-X open-tubular, 50 m × 0.5 mm stainless steel; 180°; 3 lb./sq. in. N_2;

(2) BDS open-tubular, 50 m × 0.5 mm stainless steel; 190°; 5 lb./sq. in. N_2;

(3) DEGS support-coated open-tubular, 16 m × 0.5 mm stainless steel; 180°; 2 lb./sq. in. N_2.

These columns were purchased from Perkin Elmer Ltd., Beaconsfield.

Results and discussion

Derivatives of C_{18} unsaturated alcohols were separated on the three columns and Type II separation factors calculated from the retention data. These factors are given in Table I. With each of the columns used there is a decrease in the separation factors in the order: acetate, TFA, TMS. Although the 18:1, 18:2, and 18:3 derivatives were separated from each other on all the columns there was a very poor separation of the TMS 18:0,18:1 pair on the low-polarity stationary phases.

TABLE I

TYPE II SEPARATION FACTORS FOR DERIVATIVES OF C_{18} UNSATURATED ALCOHOLS

Type II factor	*Derivative*		
	Acetate	*TFA*	*TMS*
		EGSS-X	
3/6	1.30	1.29	1.27
3/9	1.64	1.59	1.50
6/9	1.26	1.23	1.18
		BDS	
3/6	1.26	1.25	1.22
3/9	1.48	1.48	1.38
6/9	1.19	1.18	1.14
		DEGS (SCOT)	
3/6	1.25	1.19	1.16
3/9	1.48	1.41	1.34
6/9	1.17	1.16	1.14

TABLE II

EQUIVALENT CHAIN LENGTHS OF DERIVATIVES OF UNSATURATED ALCOHOLS SEPARATED ON EGSS-X

Alcohol	*Equivalent chain length*		
	Acetate	*TFA*	*TMS*
18:0	18.00	18.00	18.00
18:1ω9	18.54	18.41	18.38
18:2ω6	19.17	19.06	18.90
18:3ω6	19.68	19.54	19.37
18:3ω3	20.05	19.87	19.63
18:4ω3	20.60	20.34	20.05
20:1ω9	20.48	20.37	20.38
20:2ω9	20.98	20.76	20.60
20:2ω6	21.12	21.00	20.89
20:3ω9	21.36	21.19	21.01
20:3ω6	21.59	21.43	21.26
20:3ω3	21.96	21.74	21.51
20:4ω6	22.08	21.88	21.62
20:4ω3	22.48	22.24	21.94
20:5ω3	23.01	22.66	22.27
22:1ω9	22.36	22.31	22.36
22:5ω3	24.81	24.50	24.15
22:6ω3	25.16	24.86	24.50

The effect of the terminal group on the retention of derivatives of an extended series of unsaturated alcohols on the most polar column (EGSS-X) is shown in Table II. The best separations were achieved using the acetyl derivatives. Some reversals in retention sequence were observed: 18:4ω3 acetate is eluted after 20:1ω9, the corresponding TFA derivatives are almost coincident, and the 18:4ω3 TMS is eluted before the 20:1ω9; 20:5ω3 acetate and TFA are eluted after the corresponding 22:1ω9 derivatives, but the 20:5ω3 TMS is eluted before the 22:1ω9 derivative.

The use of TFA or TMS derivatives for the gas chromatographic separation of long-chain alcohols leads to shorter retention times but there is also a loss in separation compared to the acetyl derivatives.

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CHROM. 3897

Gaschromatographische Trennung von 2-Methyl- und 3-Methylalkanen an gepackten Säulen

Die Trennung von 2- und 3-Methylalkanen an gepackten Säulen ist, trotz vielfacher Bemühungen anderer Arbeitskreise[1–5], wegen ähnlicher Retentionszeiten beider homologen Reihen bis jetzt misslungen. Nach ŠORM[3] und Mitarbeitern wird der Peak eines Gemisches aus 2- und 3-Methylalkanen lediglich in Richtung der Retentionszeit der stärker vertretenen Komponente verschoben. Nur unter extremen Bedingungen gelang es STREIBL UND KONECNY[6] 2- und 3-Methylalkane mit Hilfe der Kapillarchromatographie zu trennen.

Uns glückte erstmalig die Auftrennung von 2- und 3-Methylalkanen an einer gepackten Säule. Als Säulenmaterial diente Chromosorb P mit 5 % Apiezon L. Die beiden homologen Reihen werden—erprobt an 1:1 und 1:2 Mischungen der Standardsubstanzen 2- und 3-Methylpentadecan, -heptadecan, -nonadecan, -heneicosan und -tetracosan—deutlich getrennt. Das Verfahren lässt sich erfolgreich auf natürliche Pflanzenwachse übertragen. Nach unseren Ergebnissen enthält das Blattwachs von *Rosmarinus off.* L. neben verschiedenen Kohlenwasserstoffreihen, über die an anderer Stelle noch berichtet wird, die 2- und 3-Methylalkane im Bereich von C_{16} bis C_{36}. Besonders deutlich verläuft die Trennung im Bereich von C_{16} bis C_{26}. Bei den geradzahligen Isokohlenwasserstoffen des Rosmarinwachses handelt es sich in diesem Bereich um ein Gemisch aus 2- und 3-Methylalkanen, bei denen das 3-Methylalkan dominiert. Die ungeradzahligen Isokohlenwasserstoffe enthalten ebenfalls beide

homologen Reihen, jedoch dominieren hier die 2-Methylalkane. Eine Ausnahme macht der C_{22} Isokohlenwasserstoff, bei dem das 2-Methylalkan vorherrschend ist. Bei den Isokohlenwasserstoffen C_{27} und C_{28} des Rosmarinwachses sind dagegen nur noch Schultern zu erkennen. Ab C_{29} haben wir keine Trennung in 2- und 3-Methylalkane mehr erreichen können. Bei den höheren Isokohlenwasserstoffen dürfte demnach das zweite Homologe entweder nur noch in so geringer Menge vorliegen, dass seine Trennung nicht mehr erkennbar ist, oder es fehlt vollständig. Auf Grund der Feststellung auch anderer Autoren[1,4,5,7] müssen wir annehmen, dass ab C_{29} bei den geradzahligen Isokohlenwasserstoffen nur noch 3-Methylalkane und bei den ungeradzahligen nur noch 2-Methylalkane vorliegen.

Experimenteller Teil

Die gaschromatographische Trennung der Alkane erfolgte mit dem Fraktometer F 6/4 HF der Firma Perkin-Elmer. Kupfersäule 6 ft.; Säulenfüllung 5 % Apiezon L auf Chromosorb P 100–120 mesh; Säulentemperatur 220° bis C_{24}, 240° bis C_{28} und 280° ab C_{29}; Gasdurchflussgeschwindigkeit 20 ml/min; Trägergas N_2; F.I.D.-Detektor; Einlasstemperatur 320°.

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CHROM. 3892

Gas chromatographic determination of morphine and cocaine in urine

Recent British legislation[1] has necessitated the establishment of treatment centres for persons dependent on diacetylmorphine and cocaine. In the U.S.A., drug monitoring systems based on thin-layer chromatographic analyses of urine have superceded the nalorphine test[2,3] but methods reported[3–5] give qualitative or semi-quantitative excretion data only. Recent gas chromatographic methods have been described for the identification and, in some cases, the determination of morphine[6–8] and cocaine[8,9] in biological fluids but our method is more sensitive, accurate and rapid and, therefore, preferable in routine measurements of the urinary output of these compounds. Data obtained from such assays may be useful in the objective assessment of dose requirements in drug-dependent persons.

Experimental

Apparatus

A Perkin Elmer Model F11 (Mark 1) dual glass column gas chromatograph with flame ionisation detector and a Leeds and Northrup 'Speedomax W' 2.5 mV recorder was used. Support coating was effected by a filtration technique[10] and the packing dried in a Hi-Eff* fluidiser[11]. Columns (1 m × 3 mm internal diameter) were packed with Chromosorb W-AW-DMCS, 100/120 mesh, coated with OV-17** (2.9%). Glass injection liners were used with "hot zone" injection.

Reagents

Diethyl ether (Analar) washed successively with sodium hydroxide solution (5% aqueous) and water, then dried over calcium chloride, and re-distilled freshly.

Chloroform (purified by passing through silica gel and alumina) to which ethanol (3%) was added.

Strong solution of ammonia, concentrated hydrochloric acid, ammonium sulphate and sodium bicarbonate, all Analar grade.

Solutions of benzhexol hydrochloride and nalorphine hydrobromide in distilled water (≡ 1 mg base/100 ml).

Bis(trimethylsilyl) acetamide (BSA) (Applied Science Laboratories Inc.) diluted, as required, 1 in 4 with dried, re-destilled carbon tetrachloride. Care must be taken to avoid contact with rubber from which BSA removes certain components, one of which has a retention time, on OV-17 columns, identical with that of morphine trimethylsilyl ether.

Procedure

Morphine. To each sample of urine (5 ml) was added concentrated hydrochloric acid (0.1 ml), nalorphine hydrobromide solution (1 ml) and ammonium sulphate (4 g): the mixture was shaken for 5–10 min. Ether was added portionwise (3 × 5 ml) and extraction achieved by shaking each time for 5 min, centrifuging and pipetting

* Applied Science Laboratories Inc., per Kodak Ltd., Liverpool.

** OV-17 is a phenyl substituted dimethylpolysiloxane polymer manufactured by Supelco Inc., Bellafonte, Pa. (U.S.A.).

off the ether layers which were rejected. Strong solution of ammonia (0.3 ml) was added to adjust to pH 9 (approx.) and further ether extraction (3 × 5 ml) was carried out. The bulked ether solution was evaporated to dryness, in a stream of nitrogen, at 45° and the residue dissolved in the diluted BSA reagent (100 μl). The solution (2 μl) was injected on to the column.

Cocaine. To each sample of urine (5 ml) was added concentrated hydrochloric acid (0.1 ml) and benzhexol hydrochloride solution (1 ml). Preliminary ether extraction was carried out (3 × 5 ml) rapidly and the ether rejected. Saturated solution of sodium bicarbonate (2 ml) was added to the aqueous layer to give pH 8 (approx.) and further ether extraction, followed by evaporation, was performed as above. The residue was dissolved in chloroform–ethanol (100 μl) and the solution (2 μl) chromatographed.

Results

Typical chromatograms, obtained under the conditions noted, are illustrated in Fig. 1. Values for the ratios of peak heights morphine TMSi ether:nalorphine TMSi ether and cocaine:benzhexol were plotted against concentrations for samples of urine to which morphine or cocaine, respectively, had been added. Linear regressions were obtained for duplicate determinations at several points over the ranges

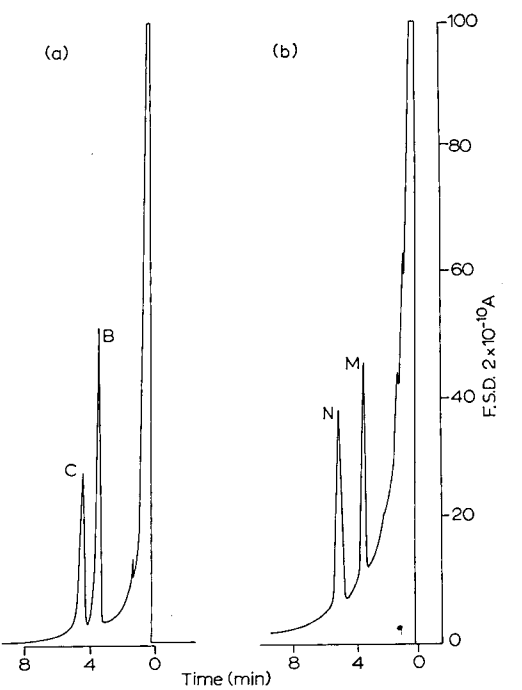

Fig. 1. Typical gas chromatograms of: (a) Cocaine, C, and benzhexol (internal standard), B. Conditions: oven, 185°; injection block, 250°; nitrogen (oxygen free), 53 ml/min; air, 40 p.s.i.; hydrogen, 23 p.s.i.; chart speed, 15 in./h. (b) Morphine trimethylsilyl ether, M, and nalorphine trimethylsilyl ether (internal standard), N. Conditions: as above, except oven 205°.

0.5–10 μg/ml (morphine) and 0.5–15 μg/ml (cocaine). Ten determinations were made at points from the upper and lower parts of each range and standard deviations for these are given in Table I.

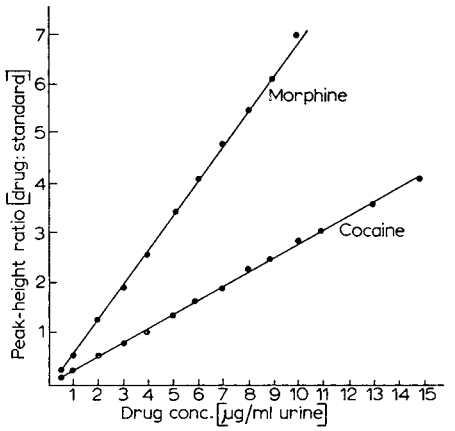

Fig. 2. Standard curves for peak height ratios of morphine trimethylsilyl ether:nalorphine trimethylsilyl ether (internal standard) and cocaine:benzhexol (internal standard) *vs.* concentrations of morphine and cocaine, respectively, added to urine.

TABLE I

REPRODUCIBILITY OF THE GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF MORPHINE AND COCAINE EXTRACTED FROM URINE

Compound	*Concentration (μg/ml)*		*Number of determinations*
	Actual	*Found* (± *S.D.*)	
Morphine	1.0	0.92 ± 0.02	10
Morphine	9.0	9.01 ± 0.15	10
Cocaine	3.25	3.01 ± 0.09	10
Cocaine	13.0	12.91 ± 0.39	10

Discussion

In any routine monitoring of drug excretion the analytical method should be as rapid and simple as possible consistent with accuracy and specificity. Gas chromatography offers a more accurate means of quantifying results than thin-layer chromatography and has advantages of speed, specificity and sensitivity as compared with spectrophotometric determinations of morphine[12–15] and cocaine[16–18] extracted from biological media.

Blood concentrations are more meaningful than excretion data in terms of drug effects and blood is easier to extract than urine, giving relatively clean solutions which resolve well on GLC analysis[8]. However, where several successive samples are necessarily required from drug-dependent persons in order to establish their individual excretion profiles over a period of time, willing co-operation of the donors is essential and urine is easier to obtain than blood!

Numerous combinations of solvents have been tried for the extraction of morphine from urine but the use of ether alone has obvious practical advantages[7]. It is an excellent solvent for many basic drugs although difficulties have been encountered with the amphoteric compounds morphine and nalorphine[19,20], recoveries of morphine from aqueous solution (pH 8–8.5) being only about 50%[7]. However, CURRY[21] and KRAMARENKO[22] recommended prior saturation of urine with ammonium sulphate and, using this technique, we have achieved upwards of 80% extraction. Conflicting reports have appeared on the efficiency of ether as a solvent for cocaine[23,24] but our experience confirms that good recoveries are obtained without prior saturation[22]. However, rapid extraction and minimal contact with acidic solutions is essential to prevent loss of cocaine, by hydrolysis, in aqueous media.

The gas chromatographic behaviour of morphine has been reported by numerous authors[7,8] and good peak shape for small amounts of free base have been obtained. Particularly with small amounts of base quantitation is facilitated by the use of trimethylsilyl ethers which give improved peak shape and eliminate sorption–desorption equilibria[25]. Using BSA as the silylating reagent gives the advantages of immediate reaction and the absence of ammonium chloride as a reaction product. Acetylation to diamorphine prior to chromatography is also advantageous[6] but this process is relatively time consuming.

Morphine, both free and conjugated, is excreted in the urine of persons taking either morphine or heroin, the amounts of free base varying in different individuals from 1–14% of the dose ingested[12]. Our lower limit of detection (0.5 μg/ml) is well below the amounts excreted even by patients receiving therapeutic doses of morphine[14] and the method has been applied successfully in analysing the urine of persons dependent on heroin taken alone or in combination with other drugs including cocaine, pethidine, methadone, methaqualone and diphenylhydramine.

Previously reported gas chromatographic methods for the detection of cocaine in urine have not been quantitative and, although there is a report on excretion of ecgonine by coca chewers[26], there are no data on the disposition of cocaine or its metabolites in humans taking the drug parenterally. Animal studies have shown marked species variation in metabolism of cocaine but it is thought that the 1–12% excretion of unchanged drug, over 24 h, in dog may be paralleled in man[27]. On this assumption our procedure is adequate to detect and estimate the drug in urine of cocaine-dependent individuals.

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CHROM. 3908

A simple constant head device

Throttled gravity flow from a reservoir is a common means of feeding eluent to chromatographic columns. If the head is great enough, the variation in flow rate caused by lowering of the liquid level in the reservoir is not serious. However, in applications such as gel filtration, when gels with high molecular weight exclusion limits are employed, a high head is likely to compress the gel bed so that required flow rates cannot be maintained. In these cases, when a low head is desirable, some form of constant head device is required to maintain a reasonably constant flow of eluent.

The Mariotte bottle (after the seventeenth century French physicist, EDMÉ MARIOTTE) is probably the most widely used constant head device but has some disadvantages. The most serious of these are sensitivity to ambient temperature changes and a tendency to surge at low flow rates and low liquid level. (See CUTLER[1] for description).

The device depicted in Fig. 1 and described in the legend has been found to be

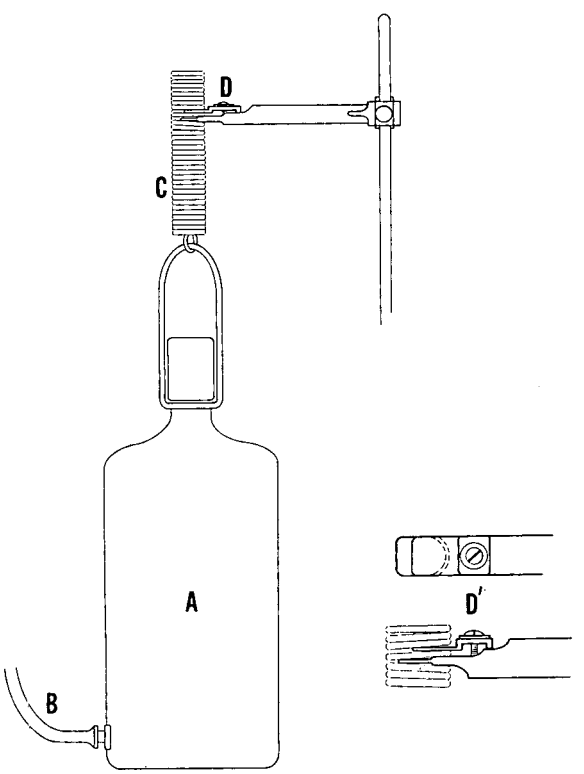

Fig. 1. Constant level reservoir: (A) reservoir with wire yoke for suspension; (B) flexible outlet tube; (C) suspension spring; (D) suspension clamp. D′ is a detail of the suspension clamp showing the groove in the lower part that serves to hold the spring in position during adjustment.

capable of maintaining a constant head ± 1 mm when assembled from readily available springs and bottles. If required, even this small variation could be reduced by refinements such as the use of an accurate cylinder as a reservoir and protection from drafts which might cause oscillation.

The spring used should be long enough and strong enough so that its elastic limit is not approached when fully loaded. The strength and length should also be such that the extension by the required load is somewhat greater than needed. The adjustment required to maintain a constant head is made at the suspension clamp. The length of spring required to match the spring extension to changes in liquid level in the suspended reservoir is first approximated by clamping an appropriate coil. Fine adjustment is then made by rotating the coil through the loosened clamp. The adjustment can also be used to compensate for solution density. Tension springs of this type are normally wound with tightly packed coils and the weight of the empty reservoir may not be enough to cause any extension. In such a case, the spring must be pre-stretched beyond its elastic limit sufficiently to slightly loosen the coils.

The outlet tube should be small and flexible with a freely suspended loop of sufficient length to accommodate the vertical movement of the reservoir; 1/16 in. I.D. by 1/8 in. O.D. vinyl tube has been found satisfactory for most applications. During calibration the tube should be suspended as it will be in use so that compensation will be included for the varying length of tube supported by the spring as the liquid level changes.

As an example, when a 1 gal. polyethylene bottle with a bottom outlet was chosen as a reservoir, it was determined that the liquid level fell 6 cm when 1 liter (1 kg) of water was withdrawn from the cylindrical portion. A spring that extended 7.5 cm/kg (0.056 in. wire, 7.5 in. long, 9/16 in. coil diameter) was then selected from the assortment available at a local hardware store. The ratio of the extension required (6 cm/kg) to the extensibility of the spring (7.5 cm/kg) multiplied by the length of the spring (7.5 in.) then gave 6 in. as the first approximation of the portion of this spring required to maintain a constant level. Final adjustment was then made by filling and draining the reservoir with the buffer to be used as eluent.

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CHROM. 3910

A refillable constant head device for continuous operation in column chromatography

The simplest method of bringing about a flow of mobile phase through a chromatographic column uses the difference in hydrostatic pressure between the liquid level above the column and that at the outflow point as the driving force. The repeated addition of small volumes of solvent to the top of the column is inconvenient and results in variable flow rates. The Mariotte bottle, or one of its modifications[1] maintains a constant hydrostatic head, thus providing both a constant flow rate and a reservoir for solvent. A stable rate of flow of the mobile phase is particularly important when a column bed of a compressible gel, such as agarose or a dextran of low cross-linkage, is used to determine the molecular size of a macromolecular substance by the measurement of its elution volume[2,3]. A disadvantage of the Mariotte bottle is that the period of uninterrupted flow of mobile phase is limited by the capacity of the solvent reservoir.

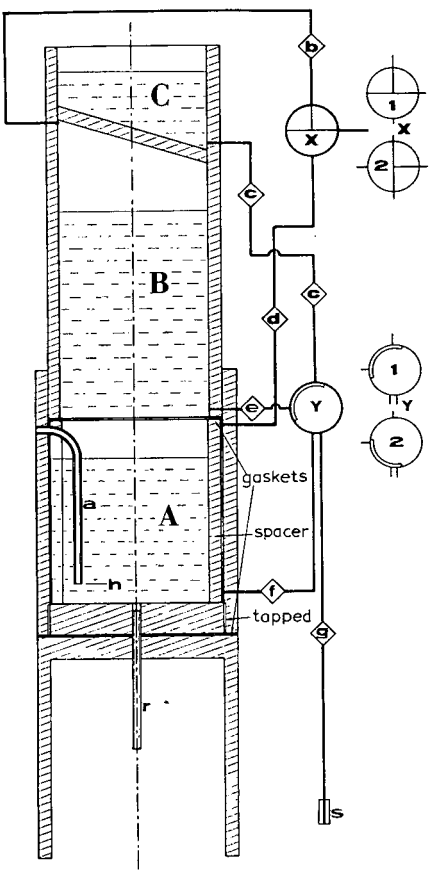

Fig. 1. Basic design of the refillable constant head device. For details, see text.

The volume of solvent which can be applied to a column at a constant rate with a variable speed peristaltic pump is not limited. Such pumps are, however, expensive and there is often pulsation and sometimes "kickback" in the solvent stream at low flow rates.

The inexpensive apparatus described here incorporates the principle of the Mariotte bottle into a device which allows the bottle to be refilled an indefinite number of times without interruption of its function or alteration in the constant head provided. This head can be selected at will. Each setting gives a completely reproducible, constant and smooth rate of flow of solvent for an indefinite period, thus combining the advantages of the Mariotte bottle with those of the peristaltic pump.

Principle

The device is shown in Fig. 1. When pipes d and f are closed by placing valves X and Y in position 1, chamber A is effectively a Mariotte bottle. This operates upon the principle that the displacement caused by fluid flow to the column via pipe r tends to cause a partial vacuum in the air space in A. Air is allowed to bubble freely into A via tube a, so that the pressure at depth h must always be equal to atmospheric. Hence the differential height between h and the column outlet is the driving force perfusing the column irrespective of the quantity of fluid in A—provided the end of tube a is covered by fluid.

The novelty of this device, and the way it differs from the simple Mariotte bottle, is the means of refilling chamber A while permitting no fluctuation in fluid head.

To maintain atmospheric pressure at h during recharging, the fluid must not be allowed to rise in tube a. This condition can be satisfied if air is removed from A at a rate exceeding that of fluid entry by the potential volume displacement which would occur in tube a if the fluid level in it were allowed to follow the rise in A. This requires a precisely regulated *net* rate of air efflux from A carefully geared to the inflow of fluid.

The critical programming of the *gross* efflux rate which this implies can be avoided in practice by removing air at any rate in excess of the required net rate, when the difference must be supplied by air bubbling into A via tube a. The efflux of air from A can be geared to the inflow of fluid by connecting both air and liquid phases A to those in a higher chamber (B). This is initiated by turning valves X and Y to position 2 (Fig. 1). Air is then automatically driven from A to B via tubes d and b as fluid flows, under gravity, from B to A via pipes e and f.

However, in order that the air flow from A to B should exceed the fluid inflow into A, the latter should be less than the fluid displacement in B. This is achieved in practice by allowing part of the fluid in B to drain from the apparatus via pipe g. This excess is reduced to a reasonable level by means of an orifice s from which the fluid can drip into a suitable container.

In practice it was found that chamber B should be full at the start of transfer to A, since any residual air in B can cause a rapid rise in the air pressure in A upon turning valve X to position 2 resulting in a transient rise of fluid in tube A. This arises by virtue of the lower viscosity of air than of liquids, but it can be readily avoided by ensuring that B is full before transfer.

Once chamber A is almost full, it can be isolated by turning valves X and Y

to position 1 which action also disconnects pipes f and g. This position is also suitable for refilling chamber B by transfer of fluid from C via pipes c and e, the gas in B being simultaneously vented to the atmosphere via pipe b. Thus chamber B can be recharged any time while A is isolated with the taps in position 1 simply by pouring fluid into C.

Equipment

A unit has been made in clear plastic so that the inside of all chambers is clearly visible (Fig. 2). The following features of the basic design shown in Fig. 1 should be noted:

(1) Since chambers A and B must be gas-tight, they are sealed by soft rubber gaskets.

(2) The whole unit may be dis-assembled by breaking one screw connection after which the spacer and plate between chambers A and B can be slid out to leave all surfaces accessible for cleaning. Since pressure must be placed upon both gaskets

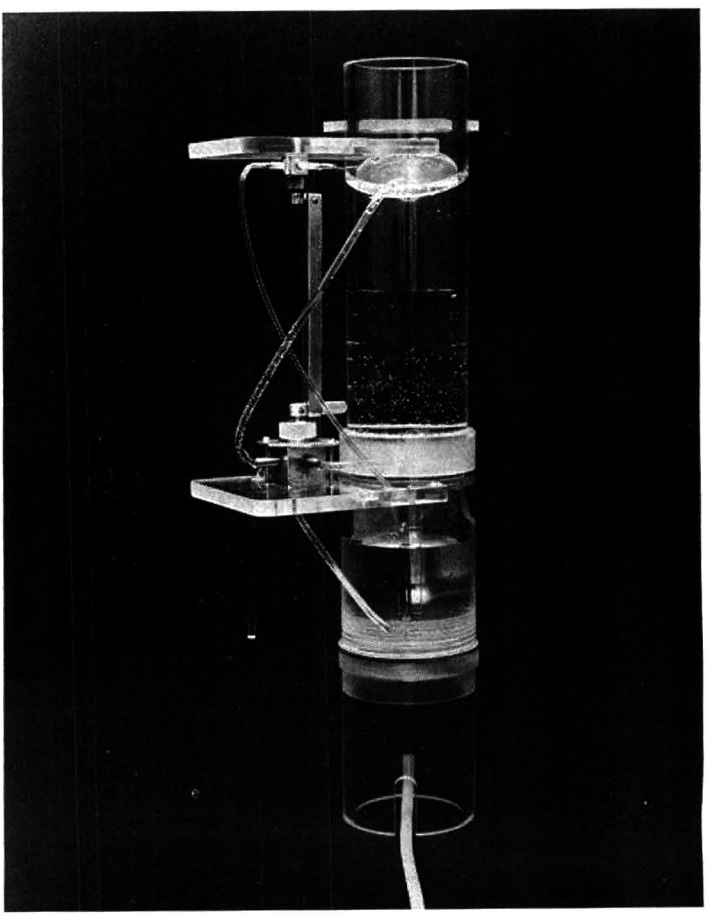

Fig. 2. Prototype of the refillable constant head device made in clear plastic.

simultaneously during assembly, the 320° cylindrical spacer must be machined to close tolerances in length.

(3) Notches cut in the lower end of tube a reduce bubble size and hence the minor fluctuations in perfusion pressures caused by bubbling in A. This modification could be incorporated equally well into the standard Mariotte bottle.

(4) The valves are mounted with a common shaft so that movement of a single lever through 90° changes both X and Y simultaneously from position 1 to 2 or vice versa. Thus there is minimal complexity in operating the unit: position 1 is used during normal running, when chamber B can be filled at any time from C, while the lever is placed in position 2 for transfer of fluid from B to A.

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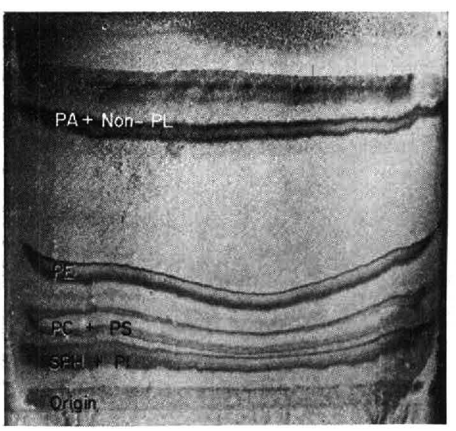
CHROM. 3902

Simplified preparative thin-layer chromatography of phospholipids

Thin-layer chromatography has proven to be a simple technique for separation of a mixture of phospholipids since one can achieve a separation in a short time[1–4]. However, a complete separation of the complex mixture of phospholipids from platelets is not attainable on a single plate. For the purpose of isolating a sufficient quantity of pure phospholipids for fatty acid analyses, we have found it necessary to rechromatograph individual bands containing a mixture of phospholipids. In order to eliminate many of the time consuming operations of rechromatography, we have evolved a modification of the basic procedure of preparative TLC.

In carrying out the modification, the usual steps for unidimensional thin-layer chromatography are followed. A lipid extract is applied as a streak along the origin of a plate (Silica Gel HR), developed with chloroform–methanol–water (65:30:5, by vol)[3]. Fig. 1 shows the separation of the major phospholipids after the first separation. The bands containing a mixture of phospholipids are viewed under an ultraviolet lamp after spraying the plate with dichlorofluorescein and the spots are outlined with a pointed instrument. The outlined areas are removed from the plate by scraping with a spatula into a beaker or test tube. A slurry is made with chloroform–methanol (5:1, by vol.) with the silica gel in a ratio of 1:1, by vol. The slurry is applied from a capillary pipette or syringe along the origin of a second plate (see Fig. 2). The application of the slurry should be such that it forms a smooth, low mound, taking care that no sharp peaks are formed on the surface. The solvent is evaporated from the mound



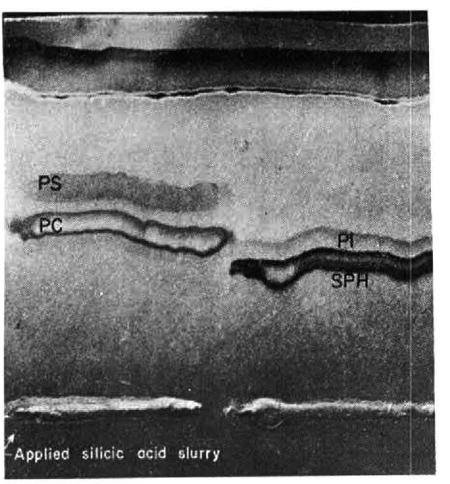


Fig. 1. Thin-layer chromatogram of a lipid extract of human platelets. SPH + PI = sphingomyelin plus phosphatidyl inositol; PC + PS = phosphatidyl choline plus phosphatidyl serine; PE = phosphatidyl ethanolamine; PA + non-PL = phosphatidic acid plus non-phospholipids. Thin layer: Silica Gel HR. Solvent: chloroform–methanol–water (65:30:5).

Fig. 2. Thin-layer chromatogram of bands SPH + PI and PC + PS taken from the thin-layer plate shown in Fig. 1 and rechromatographed. Thin layer: Silica Gel HR. Solvent: chloroform–methanol–water–HAc (80:40:5:7).

by applying a gentle stream of nitrogen over the surface. When the bottom surface of the glass above the mound returns to ambient temperature, the plate is carefully placed in a tank containing the second solvent system (chloroform–methanol–acetic acid–water, 80:40:5:7, by vol.)[5]. Fig. 2 shows the separation of the mixture of phosphatidyl choline and phosphatidyl serine into two discreet bands and a similar separation is shown for phosphatidyl inositol and sphingomyelin. Separations have been achieved with mixtures of known standards and extracts of lipids from platelets and erythrocytes, and result in essentially the same separation as is obtained by using the longer conventional procedure.

The simplified procedure has been utilized in our laboratory for the analysis of fatty acids of phospholipids by GLC. We have found that this procedure is much less laborious than the original procedure, saving several hours during each run. The length of the time saved can be substantial when a series of samples are processed.

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CHROM. 3903

Isolation of lipid hydroperoxides by preparative thin-layer chromatography of autoxidized esters of polyunsaturated fatty acids

Hydroperoxides of polyunsaturated fatty acids are the principal products in the autoxidation of lipids in biological materials. Free-radical reactions are induced by the dissociation of lipid hydroperoxides[1]. Such free-radical reactions can result in polymerization of proteins[2] and damage to biological membranes and subcellular organelles[3,4].

Pure lipid hydroperoxides, if readily available, could be important compounds in investigations of free-radical reactions involving biological materials containing lipids. PRIVETT *et al.* have prepared hydroperoxides of polyunsaturated fatty acids by either lipoxidase-catalyzed or autoxidation reactions, followed by countercurrent solvent distribution[5]. FRANKEL *et al.* developed an improved method for concentrating the hydroperoxides by liquid partition chromatography[6]. Other methods in the past have included urea fractionation[7], low-temperature crystallization[8], and adsorption column chromatography[9]. The purpose of this paper is to report a relatively simple and fast method for the preparation of hydroperoxides, in purities as high as 95 %, of polyunsaturated fatty acid esters by means of preparative thin-layer chromatography, which is an improvement over the past methods.

Experimental

Hydroperoxides were prepared from esters of polyunsaturated fatty acids by autoxidation. The esters that were used were the following: methyl linoleate, methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and ethyl esters of a fish oil fatty acid fraction obtained from menhaden (*Brevoortia tyrannus*). Individual crude samples of hydroperoxides were resolved by thin-layer chromatography.

Materials. Methyl linoleate was prepared from methyl esters of safflower oil and was purified by urea-inclusion compound fractionation, using an adaptation of the method of KEPPLER *et al.*[10]. The methanolysis method of GAUGLITZ AND LEHMAN[11] was used to prepare the esters from commercial safflower oil. A purity of 99.9 % methyl linoleate was found by a gas–liquid chromatographic (GLC) method similar to that previously published[12].

Methyl linolenate was prepared via a methanolysis reaction of linseed oil. The polyunsaturated methyl esters were concentrated by solvent crystallization and urea fractionation. The concentrate, which was analyzed by GLC, was found to contain 74.6 % methyl linolenate, 19.9 % methyl linoleate, and 5.5 % methyl oleate. This concentrate of methyl linolenate was used without further purification.

Ethyl arachidonate was obtained from Hoffmann-La Roche (Nutley, N.J.)* and was used without further purification. The sample was found to be 99.3 % pure by GLC analysis.

Methyl eicosapentaenoate was procured from Sigma Chemical Company (St. Louis, Mo.). The labeled assay was given as 90+% by the supplier. The ester was used without further purification.

* Company names are mentioned merely to simplify descriptions; no endorsement of commercial products is implied.

Ethyl esters of a menhaden oil fatty acid fraction were obtained from the U.S. Bureau of Commercial Fisheries Technological Laboratory (Seattle, Wash.). The esters were decolorized by adsorptive bleaching and were crudely fractionated by molecular distillation. This treatment removed the natural antioxidants and other non-glyceride compounds that might otherwise have been present in the oil[13,14]. The ethyl esters of the polyunsaturated fatty acids were analyzed by GLC. Table I presents the results of the GLC assay.

TABLE I

COMPOSITION OF FRACTIONATED ETHYL ESTERS OF MENHADEN (*Brevoortia tyrannus*) OIL FATTY ACIDS DETERMINED BY GAS–LIQUID CHROMATOGRAPHY

Fatty acid designation	*Percentage composition*	*Fatty acid designation*	*Percentage composition*
16:0	0.9	20:3	0.3
16:1	0.3	20:4ω6	2.6
18:0	3.3	22:1	0.5
18:1	7.5	20:4ω3	2.9
18:2ω6	0.5	20:5ω3	30.4
18:3ω3	1.4	24:1	3.0
18:4ω3	3.4	22:4ω3	2.5
20:1	2.6	22:5ω3	7.8
20:2	0.5	22:6ω3	29.6

Autoxidation procedures to prepare lipid hydroperoxides. Crude mixtures of methyl linoleate hydroperoxides were prepared by periodic shaking of a stoppered flask containing methyl linoleate (4 g) and an atmosphere of pure oxygen, first at 2–4° for a period of 2½ months and finally at room temperature for a period of 148 h. During the total period, oxygen was reintroduced into the flask five times. At the end of the first 2½ months, the peroxide value of the sample was 18.8 mequiv./kg; at the end of the total period, the peroxide value was 1,700 ± 10 mequiv./kg (27.8 % of theoretical value for methyl linoleate monohydroperoxides).

A procedure that was followed for methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and the menhaden-oil ethyl ester fraction was simpler and faster than that for methyl linoleate. This procedure involved simply placing the esters into individual Erlenmeyer flasks (50 ml size) and periodically agitating in air at room temperature until the peroxide levels reached the range of 8–25 % of the theoretical values for monohydroperoxide esters. Specifically, the final peroxide value found for each ester sample was as follows: methyl linolenate, 448 mequiv./kg; ethyl arachidonate, 1,360 mequiv./kg; methyl eicosapentaenoate, 1,280 mequiv./kg; and menhaden oil ethyl esters, 1,030 mequiv./kg.

Peroxide values. Peroxide values were determined by iodometry. The Wheeler method as modified by SWERN[15] was adapted to the determinations.

Gas–liquid chromatography. A Varian Aerograph Series 1520 gas chromatograph with a hydrogen-flame detector was used for GLC analyses. The GLC column was composed of a 179 cm by 1/8 in. O.D. stainless steel tube containing 4.0 % (w/w)

diethylene glycol succinate polyester on 90–100 mesh Anakrom SD (Analabs, Inc.). Analyses were performed after the column was conditioned overnight at 190°, with nitrogen being used as the carrier gas and flowing at the rate of 20 ml/min.

For the menhaden oil esters, conditions for temperature programming were as follows: initial column temperature 140°; program 140° to 160° at 10°/min; hold at 160° for 12 min; program 160° to 187° at 30°/min; and hold at 187°. Nitrogen carrier flow was about 10 ml/min; hydrogen flow and air flow to the detector was 25 and 200 ml/min, respectively. Electrometer range setting was at 10^{-11} A/mV. A 0–1 mV recorder was used. The volume of the sample was 0.04 μl.

Preparative thin-layer chromatography. Weighed samples (0.34–0.63 g) of autoxidized methyl linoleate were streaked on 2 mm layers of Silica Gel F_{254} (20 × 20 cm plates; E. Merck AG, Darmstadt) at a distance of 1.5 cm along one edge and about 0.5 cm from each side of the plates. The chromatoplates were then developed for a distance of 17–18 cm with a solution of petroleum ether (b.p. 30–60°)–diethyl ether (60:40, v/v). The hydroperoxide band was detected by viewing the developed chromatoplates under short-wavelength ultraviolet light. The hydroperoxides were revealed as a dark band on the phosphor-containing Silica Gel F_{254} plates. A reference chromatoplate, on which methyl linoleate and reduced methyl linoleate hydroperoxides (via $NaBH_4$ reduction) were spotted, was analyzed simultaneously with a preparative chromatoplate to aid in identification of band components.

The separated isomeric hydroperoxides of methyl linoleate were recovered by scraping the hydroperoxides from their corresponding region of the plates and extracting them with peroxide-free diethyl ether. The ether was removed by evaporating it in a stream of nitrogen at room temperature.

The autoxidized products of methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and ethyl esters of the menhaden oil fatty acid fraction were separated by preparative TLC in the same manner as that for products of methyl linoleate oxidation. The respective hydroperoxides were likewise recovered from preparative chromatoplates.

Results and discussion

In the preparative TLC separations, the lipid hydroperoxides (LOOH) were resolved at a lower R_F value than the R_F value for the non-autoxidized lipid. For instance, in the case of ethyl arachidonate hydroperoxides, LOOH (20:4), the chromatoplate produced an R_F value range of 0.68 to 0.87 for the unreacted ester and an R_F value range of 0.38–0.58 for LOOH (20:4). Multiple developments of the chromatoplates were found to improve the resolutions on plates that had high load levels, *e.g.* 0.63 g.

The recovery of the various lipid hydroperoxides ranged in quantities from 21 mg to 52 mg, depending on the amounts streaked on the plates and the initial concentrations in the autoxidized samples. Recoveries from two chromatoplates of 22.2 wt.% and 23.6 wt.% of concentrated methyl linoleate hydroperoxides, which, when combined, resulted in a product that had a peroxide value of 5,750 $\pm$ 60 mequiv./kg. Based on a theoretical value of 6,125 mequiv./kg, the methyl linoleate hydroperoxides were 94 mole % pure.

Table II gives the analytical values for the recovered lipid hydroperoxides. The peroxide values indicate a high degree of concentration of the hydroperoxides.

No attempt was made to resolve *cis-trans* and *trans-trans* conjugated dienoate isomers, which are formed as the products of autoxidation. The work of PRIVETT and coworkers[16] aptly demonstrated the fact that low temperature (*e.g.* 0°) autoxidations result in lower ratios of *trans-trans* to *cis-trans* conjugated dienes than will be obtained at room temperature or higher (*e.g.* 24°). Control of isomeric ratios is possible, therefore, by maintaining the autoxidation temperature in the optimum range for the desired isomer.

TABLE II

ANALYTICAL VALUES OF LIPID HYDROPEROXIDES ISOLATED BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY

Hydroperoxide		*Peroxide value*		$E^{1\%}_{1\,cm}$ *(isooctane)*		
Ester source	*Symbol*	*Found (mequiv./kg)*	*Theoretical*[a] *(mequiv./kg)*	*At 232–235 mμ*	*At 268 mμ*	*At 300 mμ*
Methyl linoleate	LOOH(18:2)	5,750	6,125	626 (766)[b]	29.6	8.3
Methyl linolenate	LOOH(18:3)	6,320	6,165	721 (728)[b]	63.9	4.0
Ethyl arachidonate	LOOH(20:4)	5,225	5,487	551	46	5.3
Methyl eicosapentaenoate	LOOH(20:5)	5,410	5,739	574	34.9	5.3
Menhaden oil ethyl esters	LOOH(PUFA)	4,710	5,150[c]	561	45.4	6.2

[a] Based on 100% ester monohydroperoxide.
[b] Theoretical value in parentheses based on published data for hydroperoxides with peroxide values of 6,100 mequiv./kg[17].
[c] Calculation based on GLC analysis (*cf.* Table I).

The hydroperoxides of the polyunsaturated fatty acid esters were found to be quite stable when diluted in ethanol and stored at 2–4° under nitrogen. The stability was determined spectrophotometrically by periodically measuring absorptions at 232–235 mμ and observing possible changes in $E^{1\%}_{1\,cm}$ values. For example, on three consecutive days, a $1.27 \cdot 10^{-2}$ molar solution of methyl eicosapentaenoate hydroperoxides was tested for loss of hydroperoxides by measuring $E^{1\%}_{1\,cm}$ in 98.0% (v/v) ethanol. During these days, the following $E^{1\%}_{1\,cm}$ (235 mμ) values were obtained: (1) 534, 561; (2) 533, 563; and (3) 538. These values are reasonably close to the value of $E^{1\%}_{1\,cm}$ 574, which was measured in isooctane at the beginning of the experimentation (*cf.* Table II).

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CHROM. 3881

Dünnschichtchromatographisches Nachweisverfahren zur Charakterisierung von Barbituraten mit Doppelbindungen in der C-5-Seitenkette

Die allgemein gebräuchlichen chromatographischen Nachweisverfahren für Barbiturate: Quecksilber(I)-nitratlösung (BÄUMLER und Mitarb.[1]; DEININGER[6]), Zwikker- bzw. Murexid-Reaktion (STAHL[14]) geben keinen Aufschluss über die Natur der an C-5 vorliegenden Substituenten; es handelt sich vielmehr um Gruppenreaktionen (FRAHM[7]).

Zum Nachweis von Barbituraten mit Doppelbindungen in der C-5-Seitenkette sind 0.1 % bzw. 0.05 % Kaliumpermanganatlösungen angewandt worden (CHRISTENSEN und Mitarb.[4]; LEHMANN und Mitarb.[9]; SUNSHINE und Mitarb.[15]). Bromhaltige Barbitursäurederivate können mit Wasserstoffsuperoxid/Fluorescein-Reagens charakterisiert werden (WEICHSEL[16]).

Wir fanden bei der DC von Barbituraten, dass die Verbindungen dieser Stoffgruppe, welche ungesättigte Kohlenstoff–Kohlenstoff-Bindungen in der Seitenkette aufweisen, mit Fluorescein sichtbar gemacht werden, wenn das Chromatogramm vorher mit N-Bromsuccinimid behandelt worden ist.

Material und Methode

Die verwendeten Chemikalien stammten von der Fa. E. Merck AG (Darmstadt) und waren von der Qualität "Zur Analyse". N-Bromsuccinimid wurde von der Fa. Theodor Schuchert GmbH & Co. (München) bezogen.

Die Dünnschichtplatten (Grösse 200 × 200 mm) wurden nach dem Standardverfahren nach STAHL[14] mit Kieselgel G beschichtet.

Zum Auftragen der Substanzlösungen benutzten wir eine graduierte Mikropipette mit einem Fassungsvolumen von 10 μl (Einteilung: 1 μl). Die Substanzen waren in Chloroform oder in Chloroform–Methanol–Gemisch gelöst; die Lösungen enthielten 5 μg Substanz pro μl.

Für die Betrachtung der Chromatogramme im U.V.-Licht verwendeten wir eine Camag-Universal-U.V.-Lampe; die Auswertung erfolgte im langwelligen Ultraviolett.

Fliessmittel: Chloroform–Aceton (90:10) (BÄUMLER und Mitarb.[1]).

Nachweis

Sprühlösung 1. 0.4 g N-Bromsuccinimid werden in 100 ml Eisessig gelöst.

Sprühlösung 2. 0.01 g Fluorescein werden in 100 ml 96%igem Äthanol gelöst.

Vorgang. Die Platten werden mit der Sprühlösung 1 durchfeuchtet und dann 10 Min. auf 120° erhitzt. Nach Abkühlen der Platten werden diese mit der Sprühlösung 2 besprüht. Die Auswertung erfolgt bei Tageslicht und im langwelligen Ultraviolett.

Beim Besprühen des Chromatogramms mit der Fluoresceinlösung färbt sich die Platte rosarot, während die Substanzen als weisse Flecke mit einem rosaroten Rand zu sehen sind; wenn das Chromatogramm sofort nach dem Besprühen mit Lösung 2 im U.V.-Licht betrachtet wird, sieht man die Substanzen als tiefblaue Flecke auf gelbem Grund. Nach etwa 20 Min. sind die Substanzflecke intensiv gelb gefärbt und

TABELLE I

REAKTION DER UNTERSUCHTEN BARBITURATE UND THIOBARBITURATE MIT DEM N-BROMSUCCINIMID/FLUORESCEIN-REAGENS

Synonyma[a]	*Systematischer Name*	*Reaktion mit N-Bromsuccinimid/ Fluorescein-Reagens*
Alloarbital	5,5-Diallyl-barbitursäure	positiv
Alphenal	5-Allyl-5-phenyl-barbitursäure	positiv
Amobarbital	5-Äthyl-5-isopentyl-barbitursäure	negativ
Aprobarbital	5-Allyl-5-isopropyl-barbitursäure	positiv
Axeen	5-Allyl-5-(β-hydroxypropyl)-barbitursäure	positiv
Barbital	5,5-Diäthyl-barbitursäure	negativ
Brallobarbital	5-Allyl-5-(2-bromallyl)-barbitursäure	positiv
Butalbital	5-Allyl-5-isobutyl-barbitursäure	positiv
Buthalital	5-Allyl-5-isobutyl-2-thiobarbitursäure	positiv
Butobarbital	5-Äthyl-5-butyl-barbitursäure	negativ
Cyclobarbital	5-Äthyl-5-(1-cyclohexenyl)-barbitursäure	positiv
Cyclopal	5-Allyl-5-(2-cyclopentenyl)-barbitursäure	positiv
N,N'-Dimethylnealbarbital	5-Allyl-5-neopentyl-1,3-dimethyl-barbitursäure	positiv
Dormovit	5-Furfuryl-5-isopropyl-barbitursäure	positiv
Eldoral "Heyden"	5-Äthyl-5-piperidino-barbitursäure	positiv
Eunarcon	5-(β-Bromallyl)-5-isopropyl-1-methyl-barbitursäure	positiv
Hebaral	5-Äthyl-5-hexyl-barbitursäure	negativ
Heptabarbum	5-Äthyl-5-(1-cycloheptenyl)-barbitursäure	positiv
Hexobarbital	5-Methyl-5-(1-cyclohexenyl)-1-methyl-barbitursäure	positiv
Idobutal	5-Allyl-5-butyl-barbitursäure	positiv
Inactin	5-Äthyl-5-*sek.*-butyl-2-thiobarbitursäure	positiv
Kalypnon	5-Äthyl-5-crotyl-barbitursäure	positiv
Metharbital	5,5-Diäthyl-1-methyl-barbitursäure	negativ
Methohexital	α-(±)-5-Allyl-5-(1-methyl-2-pentinyl)-1-methyl-barbitursäure	positiv
N-Methylnealbarbital	5-Allyl-5-neopentyl-1-methyl-barbitursäure	positiv
Methylphenobarbital	5-Äthyl-5-phenyl-1-methyl-barbitursäure	negativ
Narconumal	5-Allyl-5-isopropyl-1-methyl-barbitursäure	positiv
Nealbarbital	5-Allyl-5-neopentyl-barbitursäure	positiv
Noctal	5-(β-Bromallyl)-5-isopropyl-barbitursäure	positiv
Pentobarbital	5-Äthyl-5-(1-methylbutyl)-barbitursäure	negativ
Pernocton	5-(β-Bromallyl)-5-*sek.*-butyl-barbitursäure	positiv
Phenobarbital	5-Äthyl-5-phenyl-barbitursäure	negativ
Probarbital	5-Äthyl-5-isopropyl-barbitursäure	negativ
Profundol	5-Allyl-5-*sek.*-butyl-barbitursäure	positiv
Propal	5,5-Dipropyl-barbitursäure	negativ
Reposal "Novo"	5-Äthyl-5-bicyclo-(3,2,1)-2-octen-3-yl-barbitursäure	positiv
Rutonal	5-Methyl-5-phenyl-barbitursäure	negativ
Secbutabarbital	5-Äthyl-5-*sek.*-butyl-barbitursäure	negativ
Secobarbital	5-Allyl-5-(1-methylbutyl)-barbitursäure	positiv
Sigmodal	5-(β-Bromallyl)-5-(1-methylbutyl)-barbitursäure	positiv
Thiopental	5-Äthyl-5-(1-methylbutyl)-2-thiobarbitursäure	positiv
Thiotyr	5,5-Diäthyl-2-thiobarbitursäure	positiv
Vinylbitalum	5-Vinyl-5-(1-methylbutyl)-barbitursäure	positiv

[a] Soweit wie möglich wurden die von der Weltgesundheitsorganisation empfohlenen Freinamen gewählt (NEGWER[11]).

der Untergrund tiefblau; am Tageslicht dagegen erscheinen sie als rosarote Flecke auf weissem Untergrund. Die Substanzflecke sind noch nach einigen Stunden sichtbar.

Ergebnisse und Diskussion

Wir untersuchten 43 Barbitursäurederivate (darunter vier Thiobarbiturate); sie sind in Tabelle I aufgeführt. Es zeigte sich, dass alle Substanzen dieser Verbindungsgruppe, die ungesättigte C–C-Bindungen in der C-5-Seitenkette aufweisen (alken-, allyl-, bromallyl-, cycloolefin- oder vinylsubstituierte Derivate) mit N-Bromsuccinimid/Fluorescein-Reagens nachweisbar sind. Sie können auf diese Weise eindeutig von den 5,5-Dialkyl- und 5-Alkyl-5-aryl-barbituraten unterschieden werden, die mit diesem Nachweisreagens keine Flecke ergeben. Die Derivate Dormovit (5-Isopropyl-5-furfuryl-barbitursäure) und Eldoral-"Heyden" (5-Äthyl-5-piperidino-barbitursäure) verhalten sich wie die Verbindungen mit olefinischen Doppelbindungen in der C-5-Seitenkette.

Mit dem N-Bromsuccinimid/Fluorescein-Reagens können weniger als 5 μg Substanz nachgewiesen werden.

Die Thiobarbiturate ergeben schon bei der Behandlung des Chromatogramms mit der Sprühlösung 1 eine gelbe bis gelbbraune Färbung. Nach Besprühen der Platte mit Lösung 2 zeigen auch die 5,5-dialkylierten Thioverbindungen Inactin, Thiopental und Thiotyr die Reaktion der Barbitursäuren, die einen Substituenten mit ungesättigter C–C-Bindung haben.

Auch andere Arzneistoffe, die nicht zu den Barbituraten gehören, können mit dem N-Bromsuccinimid/Fluorescein-Reagens nachgewiesen werden. Hierzu gehören die Derivate des Nicotinsäureamids (Bilamid, Lytosin, Nicethamid und Nicotinamid), die Purine (Coffein, Theobromin und Theophyllin), die Pyrazolone (Aminophenazon, Antipyrinaldehyd, Isopyrin, Jodopyrin und Phenazon), die Acetanilidderivate (Antifebrin, Lactophenin, Paracetamol und Phenacetin), sowie die Wirkstoffe Salicylamid, Persedon, Methaqualon und Ethinamate. Nach COOK[5] werden auch die schwefelhaltigen Insektizide der Gruppe der Phosphorsäureester mit einer Fluoresceinlösung in 1/10 *N* Natronlauge sichtbar gemacht, wenn man das Chromatogramm vorher mit einer Lösung von N-Bromsuccinimid in Methylchloroform behandelt. Weiterhin benutzen POPOV UND GADEBA[12] das N-Bromsuccinimid/Fluorescein-Reagens zum Nachweis von Vulkanisationsbeschleunigern; sie verwenden es in fast derselben Zusammensetzung wie wir zur Charakterisierung der Barbiturate mit olefinischen Doppelbindungen in der C-5-Seitenkette.

Unsere Untersuchungen zeigten ferner, dass die Bromureide (Bromisoval und Carbromal), die Hydantoine (Mephenytoin, Pesomin und Phenytoin), die Urethane (Meprobamat und Phenprobamatum), sowie die Stoffe Bemegride, Methyprylon und Glutethimide mit dem N-Bromsuccinimid/Fluorescein-Reagens nicht nachgewiesen werden können.

Das Reagens zur Charakterisierung von ungesättigten C–C-Bindungen in der C-5-Seitenkette von Barbituraten wird nur in Verbindung mit den für diese Substanzen allgemein üblichen Nachweismethoden angewandt. Für eine sinnvolle Anwendung des N-Bromsuccinimid/Fluorescein-Reagens ist es unerlässlich die Spezifität des Barbituratnachweises mit dem jeweiligen Sprühreagens zu kennen. Nach chromatographischer Trennung wird der fragliche Stoff zuerst mit einem für Barbiturate gebräuchlichen Nachweisreagens besprüht. Beim positiven Barbituratnachweis

wird ein zweites Chromatogramm mit dem N-Bromsuccinimid/Fluorescein-Reagens behandelt, um festzustellen, ob die untersuchte Verbindung ungesättigte C–C-Bindungen aufweist.

Das N-Bromsuccinimid/Fluorescein-Reagens haben wir bei der Untersuchung von biologischem Material auf die Stoffwechselprodukte von bromallylsubstituierten Barbituraten bei Vergiftungen angewandt; diese Barbitursäurederivate werden im Körper in die 5-Acetonylderivate überführt (BOEDECKER und Mitarb.[2,3]; HALBERKANN und Mitarb.[8]; LUDWIG[10]). Sowohl das Bromallyl- als auch das Acetonylderivat kann mit der Quecksilber(I)-nitratlösung angefärbt werden, während mit dem N-Bromsuccinimid/Fluorescein-Reagens nur die Verbindungen mit dem Bromallylrest im Molekül nachweisbar sind; somit können die Abbauprodukte von den unveränderten Bromallylderivaten schnell differenziert und die durch die präparative DC isolierten Metabolite vor der I.R.-Untersuchung auf ihre Reinheit geprüft werden. Mit dem Quecksilber(I)-nitratreagens sind Spuren vom unveränderten Bromallylderivat in der Metabolitfraktion schwer feststellbar, während mit dem N-Bromsuccinimid/Fluorescein-Reagens diese auf einfache Weise nachgewiesen werden können.

Wegen der grossen Zahl der in pharmazeutischen Präparaten anzutreffenden Barbituratkombinationen dürfte—wie SAHLI und Mitarb.[13] erwähnten—kaum ein Fliessmittel zu finden sein, das eine vollständige Trennung sämtlicher Barbiturate erlaubt. Das N-Bromsuccinimid/Fluorescein-Reagens erleichtert die Suche nach einem unbekannten Barbiturat erheblich, indem es wertvolle Hinweise für den Ausschluss zahlreicher Verbindungen dieser Gruppe liefert. Daher stellt dieses Reagens in der chemisch-toxikologischen Praxis ein wertvolles Hilfsmittel dar.

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CHROM. 3901

An improved formaldehyde condensation reaction for the detection of catecholamines on silica gel thin layers*

FALCK AND HILLARP introduced a fluorescence microscopical method for the histochemical demonstration of catecholamines and 5-hydroxytryptamine in which these amines are converted into highly fluorescent end products by exposure to formaldehyde gas[1–4]. Recently, it was shown that formaldehyde condensation of these monoamines also occurs in silica gel, which permits the detection of very small quantities of these compounds by thin layer chromatography[5–7]. One of the difficulties encountered in the chromatographic analysis of these compounds is that they are labile in an oxidizing environment[5]. It is well known that both catecholamines and 5-hydroxytryptamine are readily oxidized at neutral or alkaline pH[8–10] and that silica gel is capable of promoting their conversion into pigments[5], possibly because of its content of heavy metal ions.

The purpose of this study was to elucidate the influence of this oxidation-promoting capacity of the silica gel on the yield of the fluorophores obtained from formaldehyde condensation of easily oxidized amines. When the oxidizing capacity of the thin layer was diminished by adding EDTA (10^{-3} *M*) to the silica gel or when the oxygen tension in the atmosphere was reduced by carrying out the formaldehyde condensation reaction *in vacuo*, the intensity of the light emitted by the catecholamine fluorophores was increased, while that of their 3-methoxylated derivatives and of 5-hydroxytryptamine appeared unaffected (Table I). On the other hand, when the

TABLE I

FORMALDEHYDE-INDUCED FLUORESCENCE OF BIOGENIC MONOAMINES ON A SILICA GEL THIN LAYER: MINIMUM DETECTABLE AMOUNT (μg) WITH DIFFERENT REACTION CONDITIONS[a]

Thin layers: (A) Silica gel; (B) Silica gel enriched with EDTA; (C) Silica gel enriched with EDTA, formaldehyde condensation *in vacuo*; (D) Silica gel enriched with EDTA, formaldehyde condensation *in vacuo*, and subsequent gentle borohydride treatment.

Compound	*A*	*B*	*C*	*D*
Dopamine	0.03	0.03–0.01	0.01	0.003
Norepinephrine	0.03	0.03–0.01	0.01	0.003
Epinephrine	0.1	0.1–0.03	0.03	0.01
3-Methoxytyramine	0.01	0.01	0.01	0.01
Normetanephrine	0.03	0.03	0.03	0.03
5-Hydroxytryptamine	0.01–0.003	0.01–0.003	0.01–0.003	0.01–0.003

[a] The minimum detectable amount of catecholamines after exposure to formaldehyde gas varies somewhat from one batch of silica gel to another, possibly because of variations in the metal ion content.

oxidizing capacity of the silica gel was increased by adding ferric chloride (10^{-3} *M*) no formaldehyde-induced fluorescence of catecholamines could be detected whereas that of 3-methoxytyramine, normetanephrine and 5-hydroxytryptamine was un-

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affected. The simplest explanation of these observations is that the reduction in oxidizing capacity serves to protect the catecholamines from oxidative break-down, thus permitting optimal yield of the formaldehyde-induced fluorophores. However, the increased yield of highly fluorescent products is perhaps not only a result of protecting the catecholamines from oxidative degradation but can also be ascribed to modified reaction conditions, which favours the formation of fluorophores with the highest possible fluorescence intensity (quantum efficiency). It is well known that the formation of the fluorophores takes place as a sequence of reactions, one of the steps being that of oxidative dehydrogenation[11,12], and it has recently been established that the resulting monoamine fluorophores are really mixtures of fluorophores of various colours and of varying fluorescence intensity[13]. Conceivably, the formation of each individual fluorophore in the mixture can be enhanced or suppressed by variations in the reaction conditions. This idea is supported by the finding that gentle reduction of the formaldehyde-induced catecholamine fluorophores by spraying with an aqueous solution of sodium borohydride (0.1 %) resulted in an almost 10-fold increase in the fluorescence intensity (Fig. 1). A higher borohydride concentration (1 %) abolished the fluorescence completely. Particularly high fluorescence intensity is obtained when the formaldehyde condensation reaction is carried out after the elimination of oxidation-promoting factors and followed by gentle reduction with dilute borohydride.

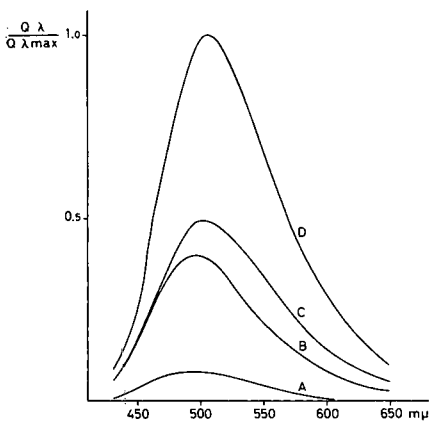

Fig. 1. Fluorescence emission spectra obtained from dopamine on a silica gel thin layer at pH 7.0 after treatment with formaldehyde under various reaction conditions. The analysis was performed in a modified Leitz microspectrograph[14] with a fixed instrument setting for direct comparison of the fluorescence intensities. For explanation of A, B, C and D, see Table I.

Whatever the explanation, it is apparent that the oxidizing capacity of the thin-layer matrix and of the gaseous environment are important factors in determining the eventual fluorescence intensity of the products formed in the condensation reaction between catecholamines and formaldehyde. By modifying the reaction conditions it should therefore be possible to increase the sensitivity of the Falck-Hillarp method and to provide a more selective demonstration of the various groups of monoamines: Anaerobic (or near-anaerobic) conditions can be used to detect both catecholamines

and indolamines, while highly oxidizing conditions reveal indolamines and 3-methoxylated catecholamines only. Apart from being of analytical chemical interest, these principles have also been applied successfully in the histochemistry of biogenic amines[15].

Experimental

Silica gel thin layers were prepared by coating standard histological glass cover slips (24 × 32 mm) with approximately 100 μ Kieselgel H (Merck, Darmstadt). The layer was applied as a slurry consisting of 20 g of silica gel suspended in 50 ml of 0.01 *M* phosphate buffer, pH 7.0. In one series of experiments EDTA (final conc. 10^{-3} *M*) was added to the slurry. The plates were dried at room temperature before used. Aqueous solutions of the amines were spotted onto the plates in volumes of about 0.5 μl. The thin layers were exposed to formaldehyde gas generated from paraformaldehyde (equilibrated in an atmosphere of about 50% relative humidity) in room air or *in vacuo* at 100° for 30 min. The resulting fluorescence was observed visually over a U.V. lamp (Sterisol, Original Hanau), equipped with a UGI filter. In some cases the plates were sprayed with an aqueous solution of sodium borohydride (0.1 %) and then again observed in U.V. light. Microspectrofluorimetric analysis was performed in a modified Leitz microspectrograph as described in a previous communication[14].

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CHROM. 3907

Polyamide–silica gel thin-layer chromatography of red food dyes

The separation of synthetic food dyes by thin layers of cellulose[1], silica gel[2], aluminum oxide[3], polyamide[4], and paper chromatography have been reported, but none of these techniques gave entirely satisfactory results. The application of a mixed polyamide–silica gel thin layer in the chromatographic separation has not been reported. In this note, the separation of eleven red food dyes and two harmful red dyes (Rhodamine B and Coralline) by this mixed layer is described.

Experimental

Preparation of polyamide–silica gel mixed layer

Seven grams of polyamide (ε-polycaprolactam CM 1007S of Toyo Rayon Co., Tokyo, Japan) were dissolved in 100 ml of 75 % formic acid. After gentle warming, a homogeneous solution was obtained; then 52 g of Silica Gel G (E. Merck) was added. Of

TABLE I

CHROMATOGRAPHIC DATA

Solvent I: isopropyl alcohol–5 % NH_4Cl solution (4:1.5). Solvent II: ether–isopropyl alcohol–5 % NH_4Cl solution (0.5:1:1). Solvent III: $CHCl_3$–isopropyl alcohol–5 % NH_4Cl solution–glacial acetic acid (0.5:2.5:1.0:0.5). Solvent IV: *n*-butanol–ethanol–5 % sodium citrate solution (3:2:1.5). Solvent V: $CHCl_3$–isopropyl alcohol–5 % NaCl solution–glacial acetic acid (0.5:2.5:0.5:0.1).

No.	*Substance*	R_F *value*[a]				
		I	*II*	*III*	*IV*	*V*
1	Ponceau 3R	0.86[b]	0.85[b]	0.71[b]	0.50[b]	0.00
2	Amaranth	0.69	0.70	0.52	0.16	0.02
3	Erythrosine	0.44	0.43	0.56	0.22	0.40
4	Ponceau SX	0.74	0.73	0.67	0.36	0.32
5	Oil Red XO	0.67	0.81	0.97	0.94	0.88
6	Ponceau 2R	0.76	0.88	0.74	0.44	0.37[b]
7	New Coccine	0.61	0.78	0.48	0.24	0.12
8	Eosine	0.55	0.58	0.65	0.31	0.44
9	Phloxine	0.57	0.66	0.69	0.34	0.35
10	Rose Bengale	0.37	0.45	0.53	0.26	0.25
11	Acid Red	0.64	0.64	0.78	0.48	0.42
12	Rodamine B	0.62	0.68	0.94	0.71	0.70
13	Coralline	0.80	0.91	0.81	0.69	0.56

[a] The R_F values are the mean of five chromatograms.
[b] Tailing.

the above-mentioned solution 200 ml were poured into a dish (14.5 × 19.5 × 2.5 cm) and a glass plate (12 × 14 × 0.1 cm) was dipped into it. Both sides of the glass were covered homogeneously. The glass was hung for 2 min over the dish to let the excess solution drain back. It was then air-dried for 3 h and heated at 100° for 30 min. These layers can be stored for a long period.

Chromatographic procedure

The standard techniques of ascending thin-layer chromatography[6] was used.

Results and discussion

R_F values obtained with five solvent systems are given in Table I. Separation is achieved by these solvent systems and the detection limit of each compound is approximately 2 μg. It has been found that small and sharp spots are obtained with the addition of salts. The time required to ascend 10 cm from origin is 3–4 h. Separation is based on the formation of hydrogen bonds between the polyamide and the sample and adsorption or partition between the silica gel and the sample. The method described gives good resolution and reproducibility. The layer did not crack or peel and can be stored easily. Both sides of the glass are independent of each other and chromatography can be performed simultaneously on both sides.

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The paper chromatography of tar polycyclic hydrocarbons

A correction of previous results

In 1957 a paper chromatographic solvent system was described which, without any previous column or other pretreatment, separated that fraction of the neutral portion of naturally occurring high-temperature tar mixtures which contains the carcinogenic polycyclic hydrocarbons[1]. Then a new kind of the semi-quantitative spot evaluation *in situ* was added, *viz.* the horizontal diameter measurement calibration[2].

But some hydrocarbons were identified incorrectly[1–3] due to the lack both of some authentic compounds and of reliable data concerning their fluorescence. In addition, the R_F values are higher when employing 45 × 45 cm Whatman No. 4 paper impregnated with a 10% petroleum ether–paraffin oil solution; this paper is thinner than the 46 × 57 cm sheets and therefore the separation is better. A characteristic paper chromatographic pattern was obtained and a revised identification of the hydrocarbons was made (Fig. 1). Previously a benzene–coal tar solution of suitable concentration had been applied. The spots were outlined over a U.V. transluminator. Thus the highest analytical sensitivity was achieved. The R_F values may vary from run to run, but the sequence of the hydrocarbons is constant. The solution was applied

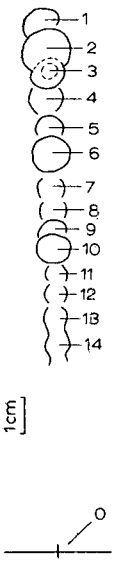

Fig. 1. R_F values, fluorescence colour, and identification of the strongly fluorescing hydrocarbons of the coal tar neutral portion fraction containing carcinogenic polycyclic hydrocarbons. (1) 0.46, violet, anthracene (the R_F value of authentic phenanthrene is 0.01 R_F unit lower; fluorescence violet, but it sublimes readily). (2) 0.44, emerald green, fluoranthene. (3) 0.42, green with violet ring, pyrene. (4) 0.39, blue-violet, chrysene. (5) 0.37, violet with touch of yellow, 1,2-benzanthracene. (6) 0.34, blue-green, 3,4-benzofluoranthene. (7) 0.32, blue, perylene with 1,2-benzopyrene. (8) 0.30, violet, 3,4-benzopyrene. (9) 0.28, violet with touch of yellow, dibenzanthracenes (1,2,5,6 and 1,2,3,4). (10) 0.26, ripe lemon yellow, 1,2,3,4-dibenzopyrene. (11) 0.24, violet, 1,12-benzoperylene. (12) 0.22, blue, anthanthrene. (13) 0.20, yellow with a touch of blue, not yet identified. (14) 0.18, yellow with a touch of blue, possibly coronene (this is unconfirmed because the standard hydrocarbon was not of a sufficient purity). O = origin.

with Kirby's automatic micropipette, 0.75 μl in capacity, with the whole volume always being applied at once to the origin (1.5 cm from the edge), the depth of the ascending mobile phase (*viz.* methanol saturated with paraffin oil) being 1.0 cm. A glass cylinder, 20 cm in diameter and 40 cm in height, with ground glass stopper, was employed as a chromatographic tank. The developing time was 5 h at room temperature.

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A note on the chromatography of phosphorus anions

Many ion exchange separations of phosphorus anions have been reported[1] and their achievement is now quite routine. We report here some interesting correlations between structure and retention time.

Separations of various mixtures of phosphorus anions were performed using continuous elution with an approximately linearly increasing concentration of KCl in water buffered at pH 11.4. It was found that the retention times were, to a very good approximation: (a) proportional to the flowrate; (b) proportional to the gradient used; (c) when the gradient was not started from zero concentration of salt, decreased by a time equal to the time which would have been taken to reach the initial concentration if the elution had started from a zero concentration.

TABLE I

RETENTION TIMES FOR VARIOUS AMIDOTHIOORTHOPHOSPHATES UNDER VARIOUS CONDITIONS OF ELUTION
All at pH 11.4; linear gradients of 1 l into 1 l.

Scheme	*Gradient (g/l)*		*Flow rate (ml/min)*	*Retention times (min)*			
	Reservoir	*Initial*		$PO_2(NH_2)_2^-$	$POS(NH_2)_2^-$	$PO_3NH_2^{2-}$	$PO_2SNH_2^{2-}$
1	30	0	1.01	40	150	250	400
2	30	5	1.01	0	0	60	140
3	50	0	1.01	20	110	165	250
4	50	5	1.01	0	0	40	80
5	100	0	1.01	10	55	90	125
6	100	0	1.33	10	40	60	90
7	150	0	1.01	10	35	55	85

Data for various flow rates, gradients and initial concentrations are given in Table I.

From the values in Table I the additivity of structural features may be seen. The series PO_4^{3-}; PO_3S^{3-}; $PO_2S_2^{3-}$; POS_3^{3-}; PS_4^{3-} occurs with equal time intervals between species (*e.g.* in row 6 they are 60–65 min apart). $POS(NH_2)_2^-$ elutes as far before $PO_2(NH_2)_2^-$ as $PO_2SNH_2^{2-}$ before $PO_3NH_2^{2-}$ but here the increment on exchanging an oxygen for a sulphur atom is only half that in the thiophosphate series. Why these different behaviours should be is not known.

Similarly with amidophosphates replacing oxygen by amide shortens the retention time by a constant (row 6:50 min).

That the additivity is a property of the fully ionized species is shown by a complete loss of such effect at pH 7.

The use of correlations such as these in predicting structures of unknown compounds is immediately obvious. For instance in reactions of P_4S_7 and aqueous Na_2S a strong peak at 540 min (scheme 6) was apparent. That this was the $P_2S_7^{4-}$ anion is likely from the retention time: $P_2O_7^{4-}$ 160 min; a PSP bridge should have little effect

not being a charged group; six terminal sulphur atoms should add 360–390 min, *i.e.* a total of 520–550 min. On isolation the compound proved in fact to be a $P_2S_7^{4-}$ salt.

Experimental

An ion exchange column 6 cm long by 1.2 cm diameter was filled with Dowex 1X10 200–400 mesh anion-exchange resin. The linear gradient was constructed by connecting with a syphon tube two similar cylindrical bottles of 1 l capacity filled with KCl solutions (A.R. grade) buffered at pH 11.4 by 20 ml of 0.88 ammonia per l. The eluent was withdrawn from the bottle of lower concentration solution whence the more concentrated solution flowed in to maintain hydrostatic equilibrium, and was well mixed. Under conditions of equal density and perfect mixing the gradient may be shown to be linear[2]. Such conditions were approximately fulfilled here. The elution of phosphorus species was continuously monitored by a Technicon Auto Analyzer assembly[3]. Sample application was in a small volume of initial eluent solution to a

					Ratio of ret. time with respect to No. 6		*Displacement due to init. conc.*	
PO_4^{3-}	PO_3S^{3-}	$PO_2S_2^{3-}$	POS_3^{3-}	PS_4^{3-}	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
390	580	860	1100	1300	4.4	4.2 ± 1		
340	300	540	780	980	4.4	4.2 ± 1	330	320
140	365	550	710	840	2.7	2.6 ± 1		
80	190	350	510	640	2.7	2.6 ± 1	200	190
325	200	280	370	440	1.32	1.37 ± 0.03		
90	145	210	270	320	1.00	1.00		
85	135	200	250	300	0.88	0.92 ± 0.03		

column which had previously been equilibrated in the same solution for half an hour or so. Between runs the column was regenerated by running 4 *N* HCl through it for about one hour. Complete reproducibility was always found.

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Chromatographie de partage sur colonne de cellulose des oxyanions du phosphore

La séparation des divers oxyanions du phosphore a été réalisée par diverses techniques chromatographiques: chromatographie sur papier, sur couche mince et sur colonne d'échangeurs d'ions[1–3]. Étant donné les excellents résultats obtenus par chromatographie sur papier ou sur couche mince de cellulose, nous avons essayé de reproduire les mêmes fractionnements sur des quantités plus grandes de produits, en utilisant les mêmes solvants en chromatographie sur colonne de cellulose[4].

Matériel et méthodes

Oxyanions utilisés

Hypophosphite monosodique	NaH_2PO_2	Schering
Phosphite disodique	Na_2HPO_3, $5H_2O$	Prolabo
Phosphate monosodique	NaH_2PO_4, H_2O	Prolabo R. P.
Hypophosphate disodique	$Na_2H_2P_2O_6$, $6H_2O$	préparé suivant le mode opératoire de BLASER ET WORMS[5].
Pyrophosphate tétrasodique	$Na_4P_2O_7$, $10H_2O$	Prolabo R. P.
Triphosphate pentasodique	$Na_5P_3O_{10}$, $6H_2O$	Progil

Mélange de polyphosphates: il s'agit d'un mélange d'orthophosphate, de pyrophosphate, de triphosphate et d'oxyanions condensés de longueur de chaîne allant jusqu'au décaphosphate, obtenus par dégradation d'un sel de Graham.

Chromatographie de partage sur colonne de cellulose

Cellulose. Nous avons utilisé la cellulose Whatman (standard grade) lavée avec HCl *M* pendant 24 h pour éliminer toutes traces de métaux alcalinoterreux et lourds. Il a en effet été montré[6] que, dans le cas de la chromatographie sur papier, les séparations sont profondément perturbées par la présence de ces métaux et qu'il était indispensable de bien laver les papiers avec des solutions acides. Nous avons éliminé l'acide par lavage prolongé à l'eau distillée jusqu'à disparition des chlorures.

Colonnes. Les colonnes (80 cm à 110 cm de long, 1.3 à 1.7 cm de diamètre) ont été remplies de cellulose lavée en suspension dans l'eau distillée. La cellulose a ensuite été équilibrée avec les solvants à la température choisie pour effectuer la séparation. Les colonnes sont chargées par 20 à 50 mg de chaque oxyanion en solution aqueuse.

Solvants. Nous avons utilisé les solvants suivants pour la chromatographie sur colonne:

Solvant alcalin: isopropanol–isobutanol–ammoniaque (20 % NH_3)–eau (40:20:1:39, v/v)[7].

Solvant acide: isopropanol–ammoniaque (20 % NH_3)–acide trichloracétique à 1000 g/l–eau (70:0.4:4:25.6, v/v)[8].

Dosage du phosphore total. Les courbes d'élution ont été obtenues en dosant les phosphates par la méthode de BRIGGS[9] dans les fractions obtenues lors de la chromatographie après élimination des alcools par chauffage à la flamme. Cette précaution est en

effet indispensable, car la présence de solvants organiques modifie la coloration et perturbe ainsi le dosage.

Dosage différentiel des phosphite, hypophosphate et orthophosphate. En appliquant la méthode précédente sans minéralisation oxydante, seul l'orthophosphate est dosé dans les fractions contenant des mélanges phosphite–orthophosphate ou orthophosphate–hypophosphate.

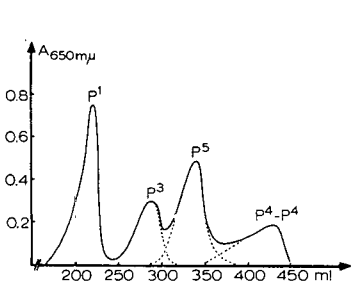
Chromatographie sur papier

L'analyse qualitative des différents oxyanions au niveau de chaque pic après chromatographie sur colonne a été effectuée par chromatographie sur papier, suivant les techniques de VOLMAR, EBEL ET BASSILI[7] et de GRUNZE ET THILO[8].

Résultats et discussion

La Fig. 1 montre le profil d'élution d'une colonne de cellulose (80 cm de long, 1.7 cm de diamètre, vitesse d'écoulement: 16 ml/h), chargée respectivement par 20 mg d'hypophosphite, de phosphite, d'orthophosphate et d'hypophosphate en solution aqueuse, et éluée à 20° par le solvant alcalin. On constate un certain empiètement des pics correspondant aux phosphite, orthophosphate et hypophosphate; la technique donne une séparation totale lorsqu'on est en présence des mélanges suivants: hypophosphite–phosphite, hypophosphite–orthophosphate, hypophosphite–hypophosphate, phosphite–hypophosphate, hypophosphite–phosphite–hypophosphate. Les séparations obtenues sont analogues à celles décrites, indépendamment de nous, par POLLARD, NICKLESS ET MURRAY[10] qui ont fractionné avec un autre solvant, également sur colonne de cellulose, le mélange hypophosphite, phosphite et orthophosphate; comme dans notre cas, la séparation entre phosphite et phosphate obtenue par ces auteurs n'est pas parfaite.

Nous avons essayé de fractionner également les polyanions condensés du phosphore par la méthode que nous avions mise au point, mais aucune séparation n'a pu être obtenue par cette technique. Ce résultat n'est pas surprenant, car il a été montré[6] que les valeurs des R_F des polyphosphates obtenues avec les solvants alcalins sont très voisines. Par contre, le mélange hypophosphite–phosphite–orthophosphate–mélange



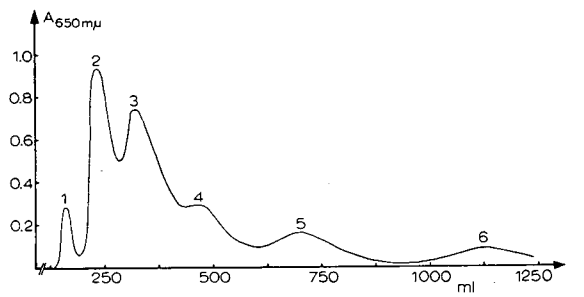


Fig. 1. Courbe d'élution en solvant alcalin du mélange hypophosphite (P^1)–phosphite (P^3)–orthophosphate (P^5)–hypophosphate (P^4–P^4) sur colonne de cellulose.

Fig. 2. Chromatographie sur colonne de cellulose en solvant acide à +4° d'un mélange d'orthophosphate et de phosphates de faible degré de condensation. Nature des pics: voir Résultats et discussion.

de polyphosphates peut être fractionné, car les valeurs des R_F des polyphosphates sont toutes inférieures à celle de l'orthophosphate.

Les résultats concernant les polyphosphates ayant été négatifs avec le solvant alcalin, nous avons essayé de les chromatographier en solvant acide qui donne d'excellents résultats en chromatographie sur papier[6]. La Fig. 2 montre la séparation d'un mélange d'oxyanions condensés et d'orthophosphate, après chromatographie sur colonne de cellulose (110 cm de long, 1.3 cm de diamètre, vitesse d'écoulement: 30 ml/h) éluée par le solvant acide à +4°. L'analyse par chromatographie sur papier (solvant acide) donne la composition suivante:

pic 1: orthophosphate;
pic 2: pyrophosphate + traces d'orthophosphate;
pic 3: triphosphate + faibles quantités d'orthophosphate et de pyrophosphate;
pic 4: tétraphosphate + ortho-, pyro- et triphosphate;
pic 5: pentaphosphate apparemment pur;
pic 6: hexaphosphate apparemment pur.

Comme le montre cette analyse qualitative des constituants des différents pics, nous n'avons pu empêcher une hydrolyse partielle des polyphosphates au cours de ce processus, malgré la réalisation de la chromatographie à +4°. C'est à partir du triphosphate que nous constatons une hydrolyse en orthophosphate et pyrophosphate, ce dernier est stable dans nos conditions. Au-delà du tétraphosphate, les pics ont une tendance à l'étalement et à l'aplatissement, ce qui provoque une dilution considérable. Au-delà de l'hexaphosphate, on n'observe pratiquement plus aucun pic.

En conclusion, la chromatographie de partage sur colonne de cellulose en solvant alcalin permet d'obtenir la séparation des oxyanions du phosphore de degré d'oxydation inférieur à 5 (hypophosphite, phosphite et hypophosphate) et de l'orthophosphate. En solvant acide, un fractionnement limité est obtenu avec les phosphates de faible degré de condensation, mais ce dernier est perturbé par l'hydrolyse concomitante de ces dérivés.

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CHROM. 3882

The study of ion pair formation

IV. Partition and ion-exchange studies of $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$

The paper electrophoretic study of ion pair formation described in two previous communications yielded a good picture of the interaction of tri-positive Co(III) complexes with various anions[1,2]. Therefore we thought that it might be interesting to examine the behaviour of such complexes in partition paper chromatography and ion-exchange paper chromatography. The results obtained are described in this note. Owing to the instability of the tris(dipyridyl) and the tris(*ortho*phenanthroline) complexes of Co(III) in most systems studied, the work deals only with the more stable $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$.

Partition chromatography

Fig. 1 shows the R_F values of the Co(III) complexes in butanol–HCl, butanol–$HClO_4$ and butanol–H_2SO_4. Little information can be obtained from these results. In butanol–HCl mixtures the R_F values increase continuously with the HCl concentration. Hydrated trivalent cations behave differently, *i.e.*, they reach a maximum

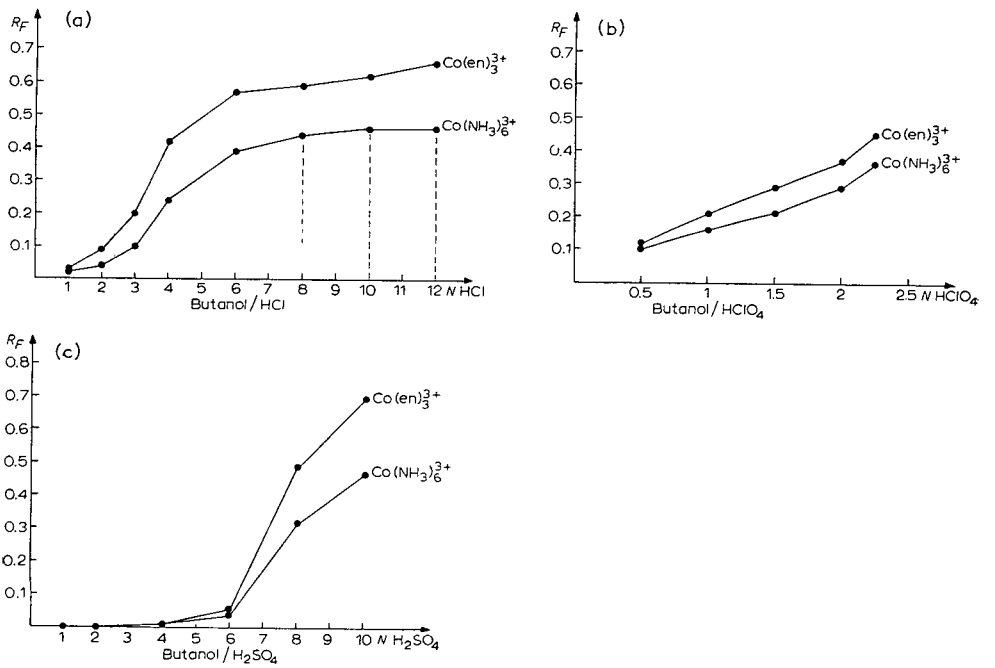
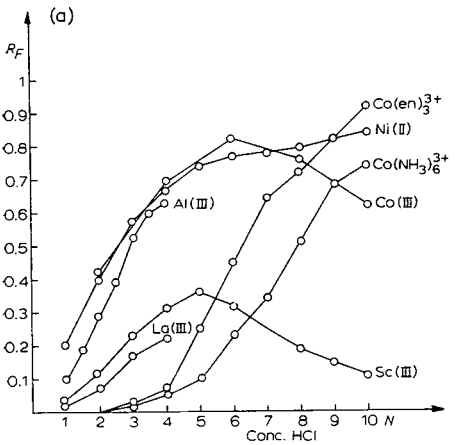
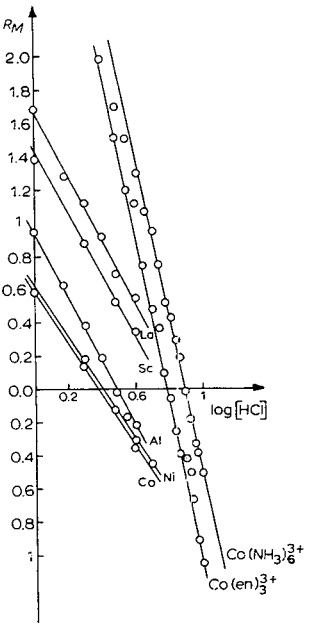
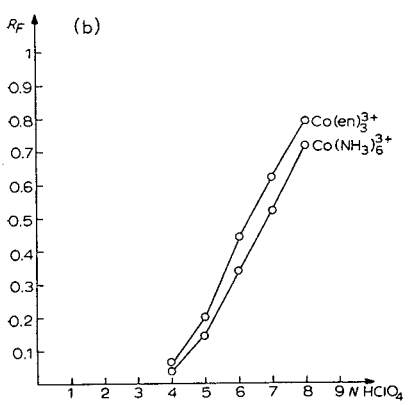
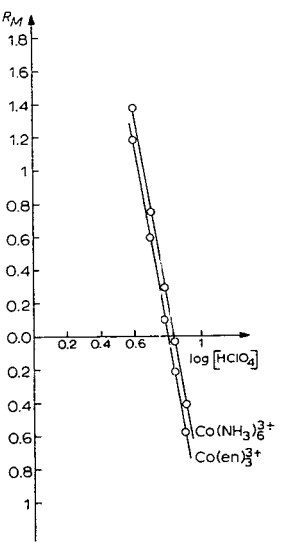


Fig. 1. R_F values of $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$ plotted against: (a) HCl concentrations in 1 : 1 mixtures of butanol and aqueous HCl. The dotted line indicates the comet formed with $[Co(NH_3)_6]^{3+}$. (b) $HClO_4$ concentrations in 1 : 1 mixtures of butanol and aqueous $HClO_4$. (c) H_2SO_4 concentrations in 1 : 1 mixtures of butanol and aqueous H_2SO_4. Whatman No. 3MM paper was used throughout.


(a)
R_F
Co(en)$_3^{3+}$
Ni(II)
Co(NH$_3$)$_6^{3+}$
Al(III)
Co(III)
La(III)
Sc(III)
Conc. HCl


R_M
La
Sc
log [HCl]
Al
Co
Ni
Co(NH$_3$)$_6^{3+}$
Co(en)$_3^{3+}$


R_F
(b)
Co(en)$_3^{3+}$
Co(NH$_3$)$_6^{3+}$
N HClO$_4$


R_M
log [HClO$_4$]
Co(NH$_3$)$_6^{3+}$
Co(en)$_3^{3+}$

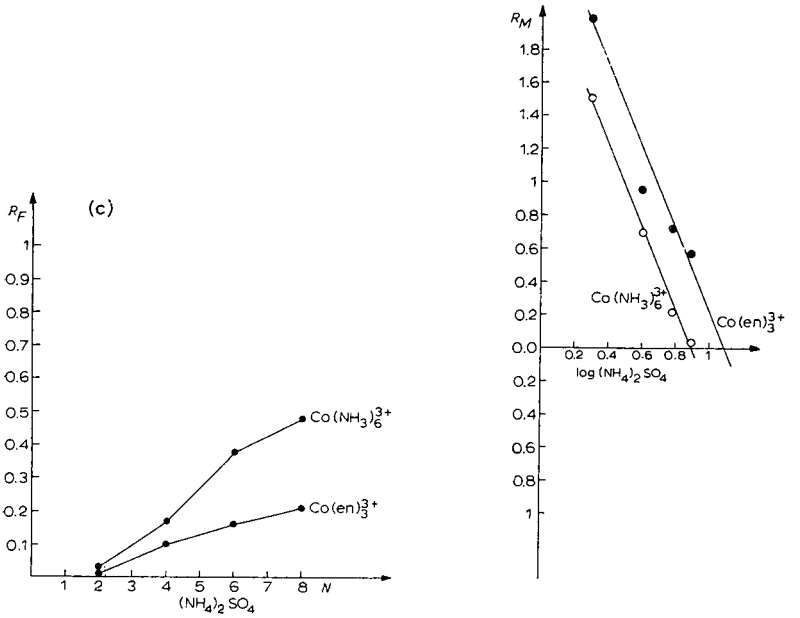


Fig. 2. (a) R_F *vs.* [HCl] and R_M *vs.* log [HCl] plots for Co(II), Ni(II), Al(III), Sc(III), La(III), $[Co(NH_3)_6]^{3+}$, and $[Co\ en_3]^{3+}$ on Amberlite SA-2 paper developed with aqueous HCl. The slopes of the R_M *vs.* log [HCl] plots are: Co(II) = 1.7, Ni(II) = 1.6, Al(III) = 2.0, Sc(III) = 1.92, La(III) = 1.94, $[Co\ en_3]^{3+}$ = 4.6, and $[Co(NH_3)_6]^{3+}$ = 4.75. (b) R_F *vs.* $[HClO_4]$ and R_M *vs.* log $[HClO_4]$ plots for $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$ on Amberlite SA-2 paper developed with aqueous $HClO_4$. The slopes of the R_M *vs.* log $[HClO_4]$ plots are: $[Co(NH_3)_6]^{3+}$ = 5.3 and $[Co\ en_3]^{3+}$ = 5.6. (c) R_F *vs.* $[(NH_4)_2SO_4)]$ and R_M *vs.* log $[(NH_4)_2SO_4]$ plots for $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$ on Amberlite SA-2 paper developed with aqueous $(NH_4)_2SO_4$. The slopes of the R_M *vs.* log $[(NH_4)_2SO_4]$ plots are: $[Co(NH_3)_6]^{3+}$ = 2.45 and $[Co\ en_3]^{3+}$ = 2.5.

around 6 *N* HCl and then decrease slowly[3]. Using butanol–$HClO_4$ no difference in behaviour between trivalent metals such as Al^{3+} or La^{3+} (ref. 4) and the Co(III) complexes is noted; the R_F values increase with the $HClO_4$ concentration. For butanol–H_2SO_4 few data are available. However, Co(II) behaves much like the Co(III) complexes. Thus these partition systems do not give any marked indication of ion pair formation except possibly in high HCl concentrations in butanol–HCl mixtures.

Ion-exchange paper chromatography

Fig. 2 shows the R_F *vs.* [eluent] and the R_M *vs.* log [eluent] plots for $[Co(NH_3)_6]^{3+}$ and $[Co\ en_3]^{3+}$ and some other ions developed with aqueous HCl, $HClO_4$ and $(NH_4)_2SO_4$ on Amberlite resin SA-2 paper (containing 45 % of a sulphonic resin).

The R_M *vs.* log [HCl] plot is analogous to the log K_d *vs.* log [HCl] plots given in column and equilibrium experiments (*e.g.*, by NELSON *et al.*[5]), and in both cases (column or paper) the slope of the line indicates the charge of the ion under study[6]. Plots of this kind are usually linear at low HCl or $HClO_4$ concentrations but become less "ideal" above approx. 3 *N* acid (see ref. 5). The trivalent metal ions exhibit a linear plot at low acidities, usually having a slope of less than 3 (2 to 2.5), which then curves upwards.

The cobalt complexes studied do not only give linear plots up to 10 *N* HCl and 8 *N* $HClO_4$, but the slopes of the linear plots are 4.7 for HCl and 5.3 for $HClO_4$, thus more than double the slope of trivalent metal ions such as La, Sc and Al.

We are tempted to explain the linearity of the plots at high acid concentration by the annulling of the usual "non-ideal" behaviour due to the increase in ion pair formation, but this explanation seems rather unlikely since paper electrophoretic data showed $HClO_4$ to form much stronger ion pairs than HCl. We have thus no satisfactory explanation for this behaviour at present. The slopes with values of approximately 5 instead of below 3 could only be explained by postulating rather strong ion pair formation between the sulphonic groups of the resin and the cobalt complexes, involving as many as three or more sulphonic groups. The slope of 2.5 obtained when developing with $(NH_4)_2SO_4$ seems to favour this explanation. Here the ion pairing with the resin and with the solution should be about equal, and the complexes should exhibit a "charge" of the order of the usual trivalent metal ions in HCl. The comparison is, however, limited because the exchanging cation was changed from H^+ to NH_4^+, and the latter is more extensively adsorbed than the former.

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Book Review

CHROM. 3850

Comprehensive Analytical Chemistry. Volume IIB. Physical Separation Methods, edited by C. L. WILSON AND D. W. WILSON, Elsevier, Amsterdam, 1968, 445 + XV pp., price Dfl. 70.

This latest volume in this series is devoted entirely to methods for the physical separation of mixtures, and contains chapters on liquid chromatography in columns, gas chromatography, ion exchangers and distillation; paper chromatography and thin-layer chromatography have had to be left to a later volume.

Over half of the chapter on *Liquid Chromatography* (J. F. K. HUBER) is devoted to theory and the remainder relates the theory with the practical side of operating liquid chromatographs, *e.g.* on the choice of adsorbent and solvent/solvent mixtures, flow rate, temperature, sample size and detector types and performance. Interest in liquid chromatography has been revived recently by the development of several new and very sensitive detectors, and this chapter is therefore a timely one: it is reasonably up-to-date (but briefly) on detectors, although Table I, 7 is little help in comparing the sensitivities of the new detectors because there is no uniformity as yet in the literature in expressing sensitivity data. There are no examples of the applications of liquid chromatography.

Gas Chromatography (E. R. ADLARD, R. STOCK AND B. T. WHITHAM) is covered in Chapter II in 150 pages, and within this severe limitation it is a reasonably comprehensive account; the theoretical section is kept short, yet it will be sufficient for most practical people. The apparatus section and the subsequent sections on solid supports, stationary phases, adsorbents (for gas–solid chromatography) and the preparation of columns are full of useful background information. The chapter then gives details of the retention volume method for identification (but does not give a strong warning of its limitations) and some brief remarks on fraction collecting for spectroscopic identification. The quantitative analysis section is brief and omits two rather useful methods of reasonable precision, namely (a) "bracketing" between two known mixtures, and (b) adding a known amount of the impurity being determined (the correct calculation for which is given by J. NOVÁK AND J. JANÁK, *J. Chromatog.*, 28 (1967) 392); this section could usefully give one or two examples showing the precision attained.

Further sub-sections on the operating procedures used for gas samples, liquid samples, solid samples and polymers usefully summarise most of the important factors. The final section on applications gives brief summaries of the literature under appropriate headings.

The chapter as a whole has rather too much detail about some less important matters (*e.g.* three drawings of 11-year old katharometer apparatus, Janák's method) and some repetition (*e.g.* in pp. 143–165); several important subjects are omitted,

such as the thermal flame ionisation detector for phosphorus compounds, support-coated open tubular columns, the Curie point pyrolyser, and the merits of on-column or preheater injection. More information should have been given on fraction collecting for spectroscopic identification, automatic preparative chromatographs and on combined gas chromatograph–mass spectrometer apparatus.

Despite the above criticisms, this chapter is a clear account of the principles and practice of gas chromatography which will undoubtedly be useful to those learning the technique or using it in their everyday work.

The chapter on *Ion Exchangers* (F. C. SAVILLE) discusses the mechanism of ion exchange, describes the experimental techniques and gives a substantial section on applications, with alphabetically-arranged subheadings. Appendix 1 gives a 20-page Table of data on commercially-available exchangers, and Appendix 2 gives detailed instructions for testing them. Both the Bibliography (Appendix 3, 307 refs.) and the References (225) are arranged with the same subheadings in alphabetical order. The subject is very clearly explained and much valuable information is given. Unfortunately, the applications section is not up-to-date, as for example in the section on amino acids, the latest reference being 1956.

After giving a 20-page section on theoretical background, the chapter on *Distillation* (G. A. DUMMETT, N. A. H. HOLT AND M. G. ROYSTON) gives a wealth of details on the experimental techniques; the first part, on simple distillation, refers to the methods of the STPTC, NBA, IP, and ASTM and describes the scope and limitations of distillation range tests; in the second part, on analytical fractional distillation, the types of fractionating columns and the ancillary equipment, are described at length; this is followed by sections on vacuum distillation, low temperature distillation, steam distillation and molecular distillation. Having reviewed the available apparatus, the authors then give a useful section on the selection of a column and on the techniques for charging and operating the still.

The book is written by several authors, and this has resulted in some repetition and lack of uniformity. In the reviewer's opinion, the book would have been better with an Introductory Chapter on general principles of chromatography, dealing with isotherms and partition, elution and displacement development, and theoretical plates and resolution. This would have avoided, for example, calculation of the number of theoretical plates in 3 different ways (*i.e.* using the peak width at half the peak height (p.27), the base width (p.72) and, on p.247, the width at 0.368 of peak height); it would also have eliminated the bulk of the repetition (of which Figs. II, 12 and III, 7 are examples) and could have led to more uniformity in the use of symbols.

The book is well produced and bound, with an adequate index and only a few typographical errors.

I.C.I., Dyestuffs Division, Manchester (Great Britain) F. R. CROPPER

News

Meetings

EUCHEM CONFERENCE ON METAL PROTEINS

An EUCHEM Conference on Metal proteins will take place from April 21–26, 1969, at Helgoland, Germany. The following topics will be dealt with:

(1) Chemistry and biochemistry of iron proteins;
(2) Metal proteins with other transition-metals, *e.g.* copper, cobalt, vanadium;
(3) Model complexes for metal proteins;
(4) Physical methods for the study of proteins (E.S.R., N.M.R., O.R.D., C.D.);
(5 Round table discussion about advantages and disadvantages of the solid phase synthesis of polypeptides.

Prof. Dr. E. BAYER, Lehrstuhl für Organische Chemie der Universität Tübingen, is Chairman of the Conference.

The provisional program as well as the preliminary registration forms may be obtained from Dr. W. FRITSCHE, Gesellschaft Deutscher Chemiker, 6000 Frankfurt (M), Postfach 119075, Germany.

CHROMATOGRAPHIC TECHNIQUES

A two day Symposium on "Chromatographic Techniques" is to be held at the Department of Chemistry and Applied Chemistry, University of Salford, Salford M5 4WT, Lancs., Great Britain on 17th and 18th April, 1969.

Speakers include

Dr. D. M. W. ANDERSON, University of Edinburgh;
Dr. J. R. CORDER, University of Swansea;
Mr. M. S. J. DALLAS, Unilever Research, Welwyn;
Mr. M. J. MAGGS, W. G. Pye and Co. Ltd., Cambridge;
Dr. R. STOCKS, Regional College of Technology, Nottingham.

Time will be available for the presentation of a number of short contributed papers. Authors wishing to present papers at the meeting should send abstracts of their proposed contributions to the symposium organisers, Dr. R. J. T. GRAHAM, Mr. J. A. NOBLE and Mr. W. E. SHARPLES, by 28th February, 1969.

INSTITUTE OF PETROLEUM

GAS CHROMATOGRAPHY DISCUSSION GROUP

Pyrolysis Gas Chromatography Sub-group

Chairman: Mr. C. E. ROLAND JONES, Vinyl Products, Carshalton, Surrey, Great Britain.
Secretary: Dr. S. G. PERRY, Esso Petroleum Co. Ltd., Esso Research Centre, Abingdon, Berks., Great Britain.

The inaugural meeting of this Sub-group was held on Tuesday, 10 December 1968, in the Lecture Theatre of the Institute of Petroleum, 61 New Cavendish Street, London W.1. at 2.45 p.m. The meeting closed at 5.30 p.m.

AGENDA

(1) Introduction.
(2) Summary of replies to the questionnaire—Secretary.
(3) Survey of pyrolysis equipment—Mr. N. B. COUPE.
(4) Rationalization of PGC: The first step. Proposals for a co-operative trial—Chairman.
(5) Open discussion of proposals.

Liquid Chromatography Sub-group

The second informal symposium to be organised by this sub-group will be held at the Royal Society of Tropical Medicine and Hygiene, 26 Portland Place, London, W.1., Great Britain, on Thursday, March 27th, 1969. The scientific programme will consist of the following items:

(1) High efficiency liquid chromatography—present state of the art, by R. P. W. SCOTT, Unilever Research Laboratory, Colworth House, Bedford.

(2) Liquid–solid chromatography on silicic acid—some new aspects, by R. J. MAGGS, Pye Unicam Ltd., Cambridge.

(3) Ion exclusion chromatography of food constituents, by F. WESLEY, Crosse & Blackwell, Ltd., London.

(4) The application of liquid chromatography in columns to the analysis of steroids and pesticides, by J. F. K. HUBER, University of Amsterdam.

(5) A panel discussion on detectors for liquid chromatography.

IDENTIFICATION OF SUBSTANCES BY PAPER AND THIN-LAYER CHROMATOGRAPHY

The 4th International Symposium on Identification of Substances by Paper and Thin-Layer Chromatography organized by the Chromatography Group of the Czechoslovak Chemical Society and Laboratorio di Cromatografia of the Consiglio Nazionale delle Ricerche (Italy) will take place in Frascati (near Rome), Italy, September 22–24, 1969. Chairmen: K. MACEK (Prague), I. M. HAIS (Hradec Králové) AND M. LEDERER (Rome).

PROGRAMME

(1) Introduction (by K. MACEK, Prague).

(2) Identification of organic substances

(a) Identification based on mobility (introduced by I. E. BUSH, Richmond);

(b) Identification based on detection (introduced by E. STAHL, Saarbrücken);

(c) Reaction before or during chromatography, formation of derivatives (introduced by M. S. J. DALLAS, Welwyn);

(d) Chromatography of decomposition products (introduced by J. GASPARIČ, Rybitví);

(e) Combination with other chromatographic principles (GC, CC, electrophoresis) (introduced by J. JANAK, Brno);

(f) Combination with other physico-chemical methods (Mass and I.R.-spectroscopy, elementary analysis, use of computers etc.) (introduced by G. SZEKELY, Basel).

(3) Identification of inorganic compounds (introduced by M. LEDERER, Rome).

Since the Symposium has a definite working programme, the number of participants will be limited to 110. In addition to a small number of invited members, priority will be given to those who intend to take part in the proceedings of the Symposium either by presenting a short original paper or by contributing to the discussion. Communications which concern the theme only marginally are not suitable.

Non-committal applications giving the title and a brief abstract or description of the paper or stating the subject on which a contribution to the discussion will be presented, should be sent by April 30th, 1969 to: Dr. KAREL MACEK, *c/o* Instituto di Chimica, Piazzale delle Scienze 5, Roma, Italy.

MEETINGS OF THE GESELLSCHAFT DEUTSCHER CHEMIKER IN 1969

March 10–15, 1969	Symposium of the Working Group Mass Spectrometry, Section Atomic and Molecular Impact Processes together with the Divisions Plasma Physics and Short Time Physics, Heidelberg.
April 8–12, 1969	Meeting on Analytical Chemistry, prepared by the Division for Analytical Chemistry of the GDCh.
April 15–18, 1969	3rd International Protactinium Conference organized by the Gesellschaft Deutscher Chemiker in cooperation with GDCh-Fachgruppe "Kern-, Radio- und Strahlenchemie", Schloss Elmau, near Mittenwald. (The number of participants is limited to 100 persons.)
April 22–26, 1969	EUCHEM Conference "Metal Proteins", Isle of Helgoland. (The number of participants is limited to 100 persons.)
May 7–10, 1969	Meeting of the Division for Coatings and Pigments of the GDCh, Bad Ems.
May 13–14, 1969	Annual Meeting of the Division for Water Chemistry of the GDCh, Bad Dürrheim (Black Forest).
September 15–20, 1969	General Assembly of the Gesellschaft Deutscher Chemiker in Hamburg.
	Meetings of Divisions on the Occasion of the General Assembly of the GDCh:
	Analytical Chemistry Division;
	Coatings and Pigments Division;
	Independent Chemists Division;
	History of Chemistry Division;
	Industrial Judicial Protection Division;
	Semiconductor Chemistry Division;
	Nuclear-, Radio- and Radiation Chemistry Division;
	Food Chemistry and Forensic Chemistry Division;
	Macromolecular Chemistry Division.

Details concerning the above mentioned events may be obtained from Gesellschaft Deutscher Chemiker, 6000 Frankfurt (M), Postfach 119075, Germany.

Appointments

Dr. NORMAN E. COOKE, principal chemical engineer for Canadian Industries Ltd., Montreal was elected President of The Canadian Society for Chemical Engineering for 1968–1969. Chosen as Vice-President of this 2,000 member national organisation was THOMAS B. DOHERTY, Imperial Oil Ltd., Toronto.

Born in Vancouver, B.C. in 1922, Dr. COOKE graduated from the University of British Columbia, and received his doctorate in chemical engineering from Massachusetts Institute of Technology.

He worked in fisheries research in Vancouver, served in Korea with the Royal Canadian Engineers, and jointed C.I.L., his present employer, in 1956.

Dr. Cooke is well known for his work in the field of pollution control and currently serves on pollution control committees of the Canadian Manufacturers Association, the National Research Council and the Canadian Standards Association. He is a member of several societies including the Engineering Institute of Canada, the American Institute of Chemical Engineers, and the Corporation of Engineers of Quebec.

In addition to Dr. COOKE and Mr. DOHERTY, the other members of the new executive are: Secretary—Dr. I. G. DALLA LANA, University of Alberta, Edmonton; and Treasurer—Dr. E. H. NENNINGER, Hatch & Associates, Montreal.

In addition, the following three new Directors were elected: Dr. G. A. RATCLIFF, McGill University, Montreal; Dr. J. P. SUTHERLAND, Chemcell Ltd., Edmonton; and Dr. N. J. THEMELIS, Noranda Research Centre, Pointe Claire, P.Q.

New Chemicals

Pierce Chemical Company, Rockford, Ill., announces Tri-Sil 'Z', a single solution silylating reagent and solvent that will rapidly and quantitatively silylate sugars and other polyhydroxy compounds. Tri-Sil 'Z' is based on a new principle and has three advanced capabilities: (1) it will silylate in the presence of moisture; (2) is fast and quantitative for difficult polysaccharides; and (3) exhibits less anomerization with monosaccharides than any method yet reported. All sugars that have been checked with this new reagent are apparently derivatized quantitatively as soon as dissolved. Experimental work thus far has been confined mainly to sugars but it is expected that many other polar compounds will respond satisfactorily to this new reagent. Tri-Sil 'Z' is packaged in convenient hypovials.

For further information apply to the publisher under reference No. Chrom. N-162.

Supelco, Inc., Bellefonte, Pa. announces the availability of several *new silicones* for use as gas chromatographic stationary phases. The most polar, OV-225, is a cyanopropyl phenyl substituted silicone which has been used successfully to 300°. This material is significantly more polar than any of the OV silicones previously offered.

The second new material OV-210 is a trifluoropropyl silicone which is selective for ketones and alcohols. It also has been used at temperatures up to 300°.

For further information apply to the publisher under reference No. Chrom. N-180.

Pharmacia Fine Chemicals AB, Uppsala, Sweden have announced the availability of QAE-Sephadex which is a fully quaternized, strongly basic ion exchanger for chromatography of proteins and other labile biological materials.

For further information apply to the publisher under reference No. Chrom. N-153.

New Apparatus

Edwards High Vacuum Ltd., Crawley, Sussex have announced the availability of a fast scanning 60° mass spectrometer. Apparently the versatile unit can be used as a conventional analyser, but can also be used in continuous analysis, *e.g.* gas chromatographically eluted fractions or adapted to a nude source instrument for "in system" analysis. For use as a gas chromatography monitor an Edwards helium separator is also offered. The mass range covered is 0–350 and the cost is around £7,500.

For further information apply to the publisher under reference No. Chrom. N-190.

CHROM. 3911

ANALYSIS OF FORMALDEHYDE BY GAS CHROMATOGRAPHY USING PORAPAK N

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(Received December 16th, 1968)

SUMMARY

Gas chromatography, at 120°, on porous polymer beads, Porapak N, can be used to analyse formaldehyde solutions. The rapid elution of formaldehyde, water and methanol with excellent peaks allows the determination of formalin liquors, as well as trace amounts of formaldehyde and methanol in water. A quantitative procedure is described and the results are the same or better than those obtained by classical methods for analysis of technical formalin.

INTRODUCTION

The use of gas chromatography in the analysis of formaldehyde–methanol–water mixtures is of great practical significance. Product testing, chemical engineering considerations of the parameters of a production line and studies of various reactions of formaldehyde with other compounds, all necessitate an accurate analytical method.

Gas chromatographic separation of formaldehyde has been the subject of a number of papers[1–15]. However, only a few of them were aimed at elucidating the analysis of formaldehyde solutions[1–6]. The main common denominator for all the above papers is the statement that polymerization of formaldehyde in the column has to be prevented by raising the column temperature to about 100°. This requirement is a complicating factor with respect to the choice of stationary phase. The recommended packings have been Tide on Fluoropak 80[1], Ethofat 60/25[3], polyethyleneglycol adipate[4], and sucrose octaacetate[2,3]. Recently, MANN AND HAHN[6] recommended the last of these and were against the use of Porapak N. We are not able to reproduce their findings.

Thus this paper deals in detail with the use of the synthetic porous polymer—Porapak N (Waters Associates Inc., Framingham, Mass., U.S.A.), which proved to be suitable for the analysis of the whole range of formaldehyde solutions. The separation of components is very sharp and the peaks of all the components are well suited for analytical purposes. Column performance remained unchanged even after one year of operation and elution times of the components remained constant throughout this period.

EXPERIMENTAL

Apparatus

The work was performed on a Carlo Erba, model C chromatograph (Milano, Italy). The instrument was equipped with a T.C.D. A glass column, 196 cm long, I.D. 0.175 cm was used and packed with Porapak N, batch 547, particle size 100/120 mesh. The carrier gas was pure argon at a flow rate of 5.6 ml/min and an overpressure of about 57 cm Hg. The column temperature was 120°, that of the vaporizer was 200° and the detector was heated to 240°. The samples were introduced with a 1 μl Hamilton micro syringe (Whittier, Calif., U.S.A.). The sample size was 0.1 μl. The identification of peaks was made by injecting pure components and making the test for the aldehyde with 2,4-dinitrophenyl hydrazine acidified with hydrochloric acid. Relative peak areas were measured with a Carlo Erba integrator, model 71, and printed out by a Kienzle printer.

Standard solutions

The solutions for calibration purposes were prepared by weighing binary methanol–water mixtures and adding water or methanol to a high-percentage formaldehyde-in-water solution prepared in our laboratory. Concentrations of components were determined according to ref. 16. The time of analysis was 12 min. The chromatogram resulting from the analysis of a formaldehyde solution is shown in Fig. 1.

The appropriate column temperature can be found from the log V_{Rm} *vs.* T^{-1} plot in Fig. 2.

Analysis of solutions

Chromatograms of the mixture under test. In routine analysis, the standard mixture

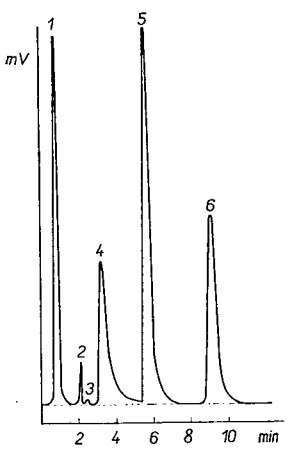


Fig. 1. A chromatogram of formaldehyde solution on Porapak N. 1 = Air; 2,3 = not identified; 4 = formaldehyde; 5 = water; 6 = methanol.

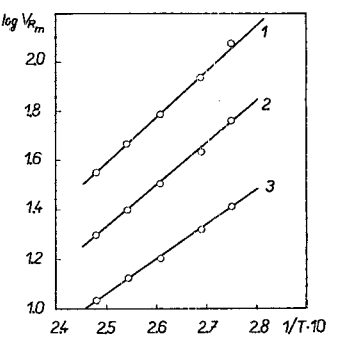
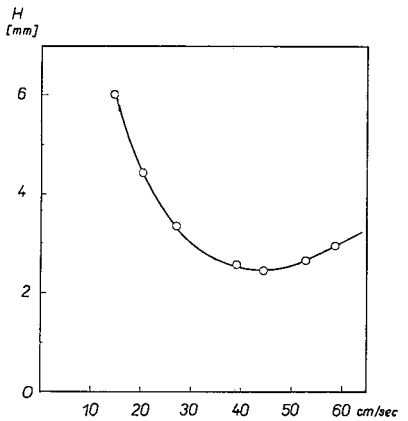


Fig. 2. The plot of log V_{Rm} against T^{-1}. 1 = Methanol; 2 = water; 3 = formaldehyde.



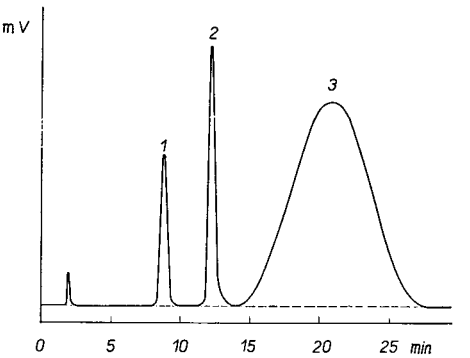


Fig. 3. The dependence of HETP on linear velocity of the carrier gas for water at 122°.

Fig. 4. A chromatogram of formaldehyde solution on a 5 m long column packed with Columnpak T + 10% Ethofat 60/25, at 115°. 1 = Methanol; 2 = water; 3 = formaldehyde.

is analysed first and then at least once again every day, as long as serial runs are made. In serial analyses it is recommended that the column is reactivated overnight at 200° at a reduced carrier gas flow, once every two weeks. Another point to be observed is that the volume of the injected mixture should be constant.

RESULTS AND DISCUSSION

The height equivalent to a theoretical plate for the above column is not particularly small as can be seen from the data for water in Fig. 3.

The column, when operated at optimum conditions, has only 800 theoretical plates per 196 cm. This number, however, will separate, in a satisfactory manner, formaldehyde, water and methanol, in contrast to a 5 m long column packed, according to ref. 4, with Columnpak T and 10% Ethofat 60/25. The latter does not give such a

TABLE I

QUANTITATIVE GAS CHROMATOGRAPHIC AND VOLUMETRIC ANALYSES OF FORMALDEHYDE SOLUTIONS

	Formaldehyde		*Methanol*	
	GC	*Titration*	*GC*	*Titration*
1	0.06	0.08	0.10	0.00
2	0.58	0.59	0.16	0.00
3	1.20	1.30	0.67	0.55
4	3.65	3.60	1.06	0.90
5	12.4	12.3	16.6	16.7
6	16.0	16.1	0.06	0.00
7	34.4	34.4	14.1	14.1
8	35.5	35.6	11.6	11.5
9	36.1	36.2	6.59	7.10
10	46.1	46.1	1.42	0.96

sharp separation and yields a very broad formaldehyde peak, as can be seen by the comparison of Fig. 3 and 4.

Another advantage of the Porapak N packed column is the short time of analysis. We found that the real elution sequence is: formaldehyde, water, and methanol. This is in contradiction with the statement by MANN AND HAHN[6] that "formaldehyde elutes after methanol and water on Porapak N".

The quantitative data summarized in Table I show that gas chromatography gives results which compare well with those obtained by the classical procedure[16] and which are reproducible. Larger deviations are only encountered with a low methanol content, thus showing that the classical method is unsuitable for the determination of methanol in formaldehyde at lower methanol concentrations. The only disadvantage to the analysis on this column is that elution of higher molecular weight components possibly present is very slow in an isothermal run and trace impurities are then very difficult to determine, particularly when present in minute concentrations.

CONCLUSIONS

Gas chromatography in a gas–solid system using Porapak N as adsorbent at 120° will separate formaldehyde, methanol and water. Rapid elution of all the components and their sharp separation permit a quantitative determination of the above compounds. The results are easily reproducible.

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CHROM. 3921

GAS–LIQUID CHROMATOGRAPHY OF RADIOACTIVE AMINO ACIDS AS THEIR TRIFLUOROACETYL ESTER DERIVATIVES

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(Received December 19th, 1968)

SUMMARY

A method is described for the gas chromatography of radioactive amino acids as their trifluoroacetylated ester derivatives. Comparative studies with the methyl, *n*-butyl and *n*-pentyl esters have been made. The absolute molar response of the flame ionization detector has been calculated for some of the derivatives.

INTRODUCTION

Using the flame ionization detector GEHRKE AND STALLING[1] analyse quantitatively twenty amino acids found in proteins by gas–liquid chromatography. Although this method is sensitive, quantitation relies on the preparation of pure standards which are used to determine relative molar responses. WATERFIELD AND DEL FAVERO[2] have described a rapid method for the purification of trifluoroacetyl (TFA) amino acid esters using liquid chromatography on silicic acid columns. Although after purification by this method the amino acid derivatives give a single peak on gas–liquid chromatography, it is not known if any of the amino acid derivatives are partially destroyed during gas–liquid chromatography resulting in the inaccurate determination of molar response factors.

In the present paper, the authors have used radioactive amino acids to investigate the synthesis of TFA amino acid esters and the possible breakdown of these derivatives during gas–liquid chromatography and to determine absolute response factors for the TFA amino acid esters using the flame ionization detector.

MATERIALS AND METHODS

Amino acids

Amino acids were supplied by the Radiochemical Centre, Amersham, Great Britain. U-^{14}C-L-aspartic acid, 6.1 mC/mmole; U-^{14}C-L-glutamic acid, 276 mC/mmole;

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U-^{14}C-glycine, 8.1 mC/mmole; 2-^{14}C-DL-hydroxyproline, 4.76 mC/mmole; U-^{14}C-DL-isoleucine, 150 mC/mmole; U-^{14}C-DL-leucine, 150 mC/mmole; L-3-phenyl-(U-^{14}C-alanine), 504 mC/mmole; U-^{14}C-L-serine, 160 mC/mmole; U-^{14}C-L-threonine, 208 mC/mmole; U-^{14}C-L-valine, 267 mC/mmole.

The radioactive amino acid (0.05 mC) was added to 25 mg non-radioactive amino acid and made up to 25 ml with water. This was stored at −20°. The specific activity of the starting solution was determined by adding 5 μl to 15 ml scintillation fluid (900 ml dioxane, 100 ml toluene, 50 ml ethanol, 60 g naphthalene, 4 g 2,5-diphenyloxazole (PPO) and 0.2 g 1,4-bis-2-[5-phenyloxazolyl]-benzene (POPOP)) in a counting vial. For all other counting the scintillation fluid consisted of 1 l toluene containing 4 g PPO and 0.1 g POPOP. The radioactivity (4,000–28,000 d.p.m.) was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314 (Packard Instrument Co. Inc., Box 428, La Grange, Ill., U.S.A.).

Preparation of TFA amino acid methyl, n-butyl and n-pentyl esters

Amino acids were esterified in dry alcoholic HCl and the esters were trifluoroacetylated by treatment with trifluoroacetic anhydride. Methyl esters were prepared by adding 2 ml redistilled dry methanol to 2 mg amino acid in a B14 test tube and dry HCl gas was bubbled continuously through the reaction mixture at 70° for 30 min. The alcohol was removed on a rotary evaporator using first a water pump and then an oil pump. The *n*-pentyl esters were similarly prepared with the reaction mixtures maintained at 108° for 30 min. The *n*-butyl esters were prepared by the interesterification procedure of GEHRKE AND STALLING[1]. The method of trifluoroacetylation was the same for all samples. To the dried ester hydrochloride residues in the test tube, 0.2 ml trifluoroacetic anhydride–dichloromethane mixture (1:4, v/v) was added and the stoppered tube was allowed to stand at room temperature for 30 min. The samples were then either made up to 10 ml in a volumetric flask with dichloromethane or the TFA amino acid ester derivatives were purified by silicic acid column chromatography[2]. Samples were stored at −20°.

Gas–liquid chromatography

A gas chromatograph Pye Series 104, Model 24, fitted with two flame ionization detectors (W.G. Pye Ltd., Cambridge, Great Britain) was used in conjunction with a Speedomax W, 1 mV recorder (Leeds and Northrup Ltd., Birmingham, Great Britain). Argon was used as carrier gas. With carrier gas flow rate 30 ml/min optimum response was obtained with air flow 700 ml/min and hydrogen 45 ml/min. Two glass columns 3.5 m × 2.5 mm internal diameter were packed with Celite 560, 72–80 mesh (acid and base washed and deactivated with dimethyldichlorosilane) and coated with 2.5% w/w of stationary phase. The stationary phase was a mixture of XE-60–QF-1–MS 200, 100 cS in the proportions 46:27:27 (w/w) respectively, developed for separating TFA amino acid methyl esters[3]. On-column injection without flash heater was made with a 10 μl syringe. The outlet of the column was connected to a stream splitter consisting of a T-piece with a 38.0 cm length of stainless steel capillary tubing (I.D. 0.3 mm) leading to the detector and a 13.5 cm length leading through a heated outlet in the oven wall to a Packard Gas Chromatograph Fraction Collector Model 850. The capillary tubing was used to increase the outlet pressure of the gas to the fraction collector thus eliminating split ratio changes during collection. The split ratio was calculated from

measurements of gas flow to the detector and fraction collector at the oven temperature for peak elution. Approximately 60% of the effluent gas was collected. Because of the low flow rate of carrier gas to the jet of the detector, the response was reduced and baseline noise on the recorder was much increased. Additional argon therefore was introduced via a capillary tube into the hydrogen gas stream, so that the total argon flow through the detector was maintained at 30 ml/min. The effluent carrier gas to the collector passed through a glass tube 4.75 × 0.75 cm packed with scintillation grade 3-p terphenyl coated with MS 550, 10% w/w (Packard Instrument Co. Inc.). The terphenyl was supported at the lower end by a 0.75 cm length of cigarette filter. During collection the upper end of the tube was pressed against a rubber seal. The tube was rapidly changed for a fresh tube by movement of the collector turntable which held 50 tubes.

To measure the trapped radioactivity, the cigarette filter and packed terphenyl were pushed by means of a glass rod through the glass tube into a counting vial containing 15 ml of scintillation fluid. The counting efficiency of the terphenyl scintillation fluid mixture was 71%. No measurable radioactivity remained in the glass tube. The values were corrected for background and for quenching.

RESULTS AND DISCUSSION

TFA amino acid esters were prepared from radioactive amino acids diluted with suitable amounts of non-radioactive amino acids and purified by liquid chromatography on silicic acid columns. A 7 μl sample of the pure derivative containing a known amount of radioactivity was injected on to the column and a known percentage of the effluent was passed through the splitter to the fraction collector. The effluent was collected during an initial isothermal period and also after the column temperature, was increased to 220° and maintained at this temperature for 20 min.

TABLE I

AMOUNT OF RADIOACTIVITY COLLECTED AFTER GAS CHROMATOGRAPHY OF RADIOACTIVE TFA AMINO ACID ESTERS

Experimental details are given in the text. 7 μl samples were injected. Isothermal conditions were maintained as shown until the peak was eluted. The oven temperature was then raised at 48°/min and held at 220° for 20 min. Trapping of radioactivity was continuous. All results are for separate experiments and are expressed as a percentage of the amount injected on to the column.

Argon passing to collector (ml/min)	*Derivative*		
	TFA valine methyl ester (initial temperature 100°)	*TFA glycine n-butyl ester (initial temperature 125°)*	*TFA aspartic acid di-n-butyl ester (initial temperature 179°)*
14.3	100	99	101
	100	100	101
	100	100	100
21.4	91	90	95
	90	92	96
	90	90	96
31.5	80	87	90
	80	87	88
	81	86	88

Table I shows the effect of carrier gas flow rate on the efficiency of trapping of the radioactive compounds. Thus at 14.3 ml/min 100% of the radioactivity was recovered for the TFA valine methyl ester but at 31.5 ml/min only 80% was obtained.

Although the radioactivity injected as the TFA amino acid ester could be recovered quantitatively at low gas flow it was found that not all the radioactivity corresponded to a single derivative peak seen on the recorder chart. When the radioactivity was collected in three separate tubes representing fractions obtained prior to the peak, during peak elution and after the peak when the oven temperature was raised to 220°, it was found that small but consistent amounts of radioactivity were eluted both before and after the peak. Table II shows such results for seven TFA amino acids as their methyl, *n*-butyl and *n*-pentyl esters. Hydroxyproline in Table II is the only amino acid consistently to show no elution of radioactive material before the peak. No explanation is offered for the 13.1% radioactivity coming off the column before the TFA phenylalanine methyl ester. It is known that phenylalanine methyl ester is more volatile than the corresponding TFA derivative, but no peak was seen on the recorder chart. All the derivatives show considerable amounts of post-peak radioactivity. The yield of TFA amino acid ester prepared under the conditions described was measured by converting a solution of amino acid of known radioactivity to the derivative. A sample of the final product was analysed by gas–liquid chromato-

TABLE II

GAS CHROMATOGRAPHY OF RADIOACTIVE TFA AMINO ACID ESTERS WITH THE RADIOACTIVITY COLLECTED BEFORE, DURING AND AFTER ELUTION OF THE PEAK

The three fractions which are expressed as a percentage of the total collected are each the average for three separate injections. The instrument details and experimental conditions are given in the text and Table I. Carrier gas flow to the collector was 14.3 ml/min.

TFA amino acid	*Ester*	*Temp. (°C)*	*R_T (min)*	*% Radioactivity collected*			*Yield (%)*
				Pre-peak	*Peak*	*Post-peak*	
Isoleucine	Methyl	118	6.3	0.0	92.6	7.4	92
Leucine		118	7.5	1.0	93.3	5.7	92
Threonine		118	6.5	1.1	94.1	4.8	75
Serine		118	11.4	5.2	89.4	5.4	74
Hydroxyproline		144	10.5	0.0	93.4	6.6	97
Phenylalanine		170	5.3	13.1	73.0	13.9	57
Glutamic acid		170	4.4	5.1	88.4	6.5	75
Isoleucine	*n*-Butyl	128	12.0	0.0	90.8	9.2	87
Leucine		128	15.5	4.7	92.2	3.1	81
Threonine		128	9.3	0.9	93.3	5.8	94
Serine		128	18.0	5.4	89.1	5.5	88
Hydroxyproline		170	7.2	0.0	88.2	11.8	88
Phenylalanine		179	7.5	2.5	89.3	8.2	85
Glutamic acid		179	14.0	2.8	90.4	6.8	84
Isoleucine	*n*-Pentyl	130	21.2	1.1	89.5	9.4	91
Leucine		130	25.0	5.1	91.9	3.0	88
Threonine		130	15.3	1.2	87.5	11.3	84
Serine		141	13.3	7.0	88.6	4.4	87
Hydroxyproline		170	11.0	0.0	89.6	10.4	93
Phenylalanine		183	9.2	4.0	80.0	16.0	69
Glutamic acid		183	21.3	8.1	79.6	12.3	67

graphy and the radioactivity was collected during elution of the peak. The percentage yield was calculated from the radioactivity in the starting solution and in the collected peak. These results are shown in the last column in Table II. For all the amino acids examined derivatization under the conditions described was incomplete and was not systematically influenced by the ester substituent. Experiments indicated that if a derivative was purified by silicic acid column chromatography prior to gas–liquid chromatography pre-peak radioactivity was less than 0.4% and post-peak radioactivity was less than 3.0%. Table III presents results for the TFA glycine and TFA aspartic acid methyl esters. It was not possible within these limits to determine whether the derivatives were incompletely purified or whether some breakdown was occurring during gas chromatography.

TABLE III

AMOUNT OF RADIOACTIVITY ELUTED BEFORE, DURING AND AFTER PEAK ELUTION OF TFA AMINO ACID ESTERS

The derivatives were purified by silicic acid column chromatography before analysis. For experimental conditions see Table II. Results are shown for six separate experiments, with the radioactivity from each 7 μl injection collected in three tubes.

% of total radioactivity collected	*Derivative*					
	TFA glycine n-butyl ester			*TFA aspartic acid di-n-butyl ester*		
	Before	*During*	*After*	*Before*	*During*	*After*
	0.2	97.9	1.9	0.2	97.1	2.7
	0.1	97.7	2.2	0.3	96.9	2.8
	0.2	97.5	2.3	0.2	96.9	2.9

The response of a gas chromatograph detector may be expressed as the quantity of electricity produced by one mole of a given compound[4]. The absolute molar response factor can be determined by injecting known amounts of a pure standard and measuring the current produced by the detector. However, it must be assumed that the standard is pure and that no destruction of the standard occurs during chromatography. Using the gas chromatograph-fraction collector system described here it is possible to determine accurately the molar response factor of radioactive compounds. This method eliminates the problem of injecting an exact and known amount on to the column. The peak area displayed on the recorder chart can be related directly to the amount of radioactivity collected during the period of peak elution. From the specific activity of the original amino acid solution and the split ratio of the emerging carrier gas the exact amount of derivative corresponding to the peak on the recorder chart can be determined. The number of coulombs produced can then be calculated if the amount of current corresponding to full scale deflection on the recorder chart is known for any particular attenuation setting of the amplifier.

Table IV shows the results for the methyl, *n*-butyl and *n*-pentyl esters of 5 TFA amino acids obtained in one experiment. The *n*-pentyl ester derivatives give the highest molar response in coulombs per mole, as expected because of their higher carbon content. They are useful for the study of a limited number of amino acids[5]. However,

TABLE IV

ABSOLUTE MOLAR RESPONSE OF THE FLAME IONIZATION DETECTOR TO TFA AMINO ACID ESTERS

Samples of TFA amino acid esters of known specific activity were chromatographed separately under isothermal conditions. Radioactivity was collected only during elution of the peaks under isothermal conditions. Gas flow to the collector was 14.3 ml/min. Full scale deflection on the recorder corresponded to 10^{-12} A for attenuation setting 1 × 1.

Amino acid	*Molar response (coulombs per mole)*		
	TFA methyl ester	*TFA n-butyl ester*	*TFA n-pentyl ester*
Glutamic acid	0.86	2.70	3.05
Hydroxyproline	1.13	1.54	2.08
Isoleucine	1.37	1.97	2.30
Serine	0.67	1.28	1.51
Threonine	0.89	1.40	1.76

all the protein amino acids have been successfully separated as the *n*-butyl[1] and methyl[3] ester derivatives using temperature programming.

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THE CHARACTERISATION OF IODOAMINO ACIDS AND THEIR DERIVATIVES BY THIN LAYER CHROMATOGRAPHY

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SUMMARY

A method of characterising iodoamino acids, *e.g.* thyroxine, iodothyronines and iodotyrosines and some of their commonly occurring derivatives, *e.g.* thyrolactic, -pyruvic and -acetic acids is described. This procedure, which is based on thin layer chromatography of the parent acids and on the preparation and chromatography of suitable derivatives, prepared in sequence, is exemplified by the characterisation of 3,3',5'-triiodothyronine, a trace metabolite of thyroxine occurring in the plasma of plaice.

INTRODUCTION

Although naturally occurring iodoamino acids are commonly separated by paper[1,2], thin layer[3,4] and gas–liquid chromatography[5,6], chromatographic behaviour of the free acids does not, in itself, constitute an adequate criterion of chemical identification.

In the course of our work on thyroid hormones in marine fauna, several species of which had previously been examined by rather inadequate techniques, it became clear that there was a need for some general procedure by which small quantities of iodoamino acids could be more rigorously identified.

The present communication describes a system of characterisation based on chromatography of the free acids and the preparation and chromatography of suitable derivatives, prepared in sequence and involving different reactive sites on the parent iodoamino acid.

EXPERIMENTAL

Chromatography

TLC plates were spread at 250 μ thickness from a slurry of silica gel (Machery Nagel, N/HR UV^{254}; 30 g) and freshly prepared rice starch solution (11.5%; 65 ml) and were air dried before use.

Samples were applied from a solution in ethanol–2 *N* ammonia (1:1) containing methyl mercaptoimidazole (0.01 *M*) as antioxidant, the spots were dried under nitrogen.

Chromatograms were irrigated, without previous equilibration, in the following solvent systems (proportions quoted are volume:volume).

(a) Chloroform–methanol–ammonia, sp.gr. 0.880 (50:25:2.5) (ref. 7).
(b) Acetic acid–methanol–ammonia, sp.gr. 0.880 (40:20:3) (ref. 7).
(c) Formic acid–methanol–chloroform, (5:15:80) (ref. 8).
(d) Ethyl acetate–methanol–ammonia, sp.gr. 0.880 (diluted 1:5) (50:20:10).
(e) Ethyl acetate–methanol–ammonia, sp.gr. 0.880 (diluted 1:5) (50:13:10).
(f) Formic acid–methanol–chloroform, (15:15:70) (ref. 8).

Spots were normally located by viewing under U.V. light; on occasions, chromatograms were sprayed with specific locating reagents for phenolic, amino[7] and ketol groupings[1,9]. Radiochromatograms were scanned by the method of OSBORN AND SIMPSON[10]. Elutions of material from the layers, prior to rechromatography, was effected with ethanol–2 *N* ammonia (1:1) containing methyl mercaptoimidazole in a conventional sinter-disc thimble.

Preparation of derivatives

Acylations were performed by dissolving the dried sample (≯ 20 μg) in aqueous sodium hydroxide (0.1 *N*; 10 μl) and adding at 0.5 h intervals two 10 μl portions of reagent (0.4% (v/v) acetic anhydride in acetone or 0.4% (v/v) benzyl chloroformate in tetrahydrofuran). After the addition of further portions of base and reagent (10 μl of each) and standing for a further period of 0.5 h the product was isolated by evaporation in nitrogen.

Hydrolyses were effected by dissolving the dried sample on aqueous sodium hydroxide (0.5 *N*; 20 μl) and allowing to stand at 0° overnight.

Carboxylic acids were converted to their methyl esters by reaction with methanol (0.5 ml) and hydrochloric acid (sp.gr. 1.18; 2 drops) overnight at 40°; the products were isolated, as before, by evaporation in nitrogen.

Methyl ethers were prepared from the parent phenols by reaction with excess diazomethane in anhydrous methanol at 0° overnight[11]. Under these reaction conditions, phenolic iodoamino acids yielded the methyl esters of the phenol ethers from which the free carboxylic acids were obtained by hydrolysis, according to the method described, acidification and extraction with ether.

Authentic compounds

3,3′-Diiodothyronine was synthesised from 3,5,3′-triiodothyronine by the method of ROCHE *et al.*[12]. The pyruvic acid analogues of 3,5,3′-triiodothyronine and thyroxine were prepared by the method of NAKANO[9]; lactic acid derivatives were obtained from them by reduction with sodium borohydride in aqueous ethanol[13]. Iodothyroacetic acids, other iodothyronines, and iodotyrosines, were obtained from normal commercial sources.

Application of characterisation procedure to biological materials

Whole blood was collected from freshly killed plaice *(Pleuronectes platessa)* which had been injected intraperitoneally with ^{125}I labelled thyroxine 24 h before

sacrifice. The plasma fraction (5 ml) was acidified (pH 2), extracted twice with *n*-butanol and the combined extracts immediately made alkaline to reduce ester formation. After the addition of methyl mercaptoimidazole (0.01 *M*; 10 μl) and appropriate inactive carriers (3,5,3′-triiodothyronine, thyroxine, 3,5,3′-triiodothyroacetic acid, 3,5,3′,5′-tetraiodothyroacetic acid; 20 μg of each) the extract was evaporated at 37° *in vacuo*, redissolved in ethanol–2 *N* ammonia (1:1, v/v), applied to a TLC plate and chromatographed in system (a). Scanning revealed the presence of several radioactive peaks, the peak of particular interest being located just behind the thyroxine carrier. This material was eluted, supplemented with authentic 3,3′,5′-triiodothyronine (T_3') (20 μg) and chromatographed successively in systems (b), (a) and (d). In all chromatograms, exact coincidence of the radio peak and authentic T_3', visualised under U.V. light, was observed. The sample was then eluted, esterified with methanol–hydrochloric acid and chromatographed successively in systems (a) and (c); exact correspondence of radiopeaks and authentic T_3' methyl ester was again observed. The esters were next eluted, saponified, acetylated and chromatographed in system (a). Two radiopeaks were observed, on scanning, coincident with O,N-diacetyl- and N-acetyl-T_3'; after elution and rechromatography in system (d), the same coincidence was observed. Elution of the diacetate, followed by hydrolysis and chromatography in system (a) furnished the N-acetyl compound coincident with authentic carrier. The monoacetates were then combined, methylated with diazomethane, saponified to release the free acid and chromatographed successively in systems (a) and (d). Exact coincidence of the radio-peak with authentic N-acetyl-O-methyl-T_3' was observed on both chromatograms.

RESULTS AND DISCUSSION

The uncertainty which is properly attached to assignments of chemical identity to compounds on the basis of their chromatographic behaviour, even in a number of

TABLE I

R_F VALUES OF SOME IODO COMPOUNDS

Compound	*System*					
	a	*b*	*c*	*d*	*e*	*f*
Thyroxine	0.13	0.45	0.27	0.38	0.29	0.50
3,5,3′-Triiodothyronine	0.22	0.37	0.19	0.48	0.33	0.41
3,3′,5′-Triiodothyronine	0.09	0.49	0.31	0.35	—	—
3,5-Diiodothyronine	0.19	0.34	0.15	0.43	—	0.34
3,3′-Diiodothyronine	0.16	0.29	0.21	0.40	—	—
3-Monoiodotyrosine	0.06	0.25	0.10	0.16	0.13	0.25
3,5-Diiodotyrosine	0.03	0.35	0.13	0.10	0.07	0.37
3-Monoiodohistidine	0.05	0.07	0.02	0.09	0.09	0.05
3,5,3′,5′-Tetraiodothyroacetic acid	0.24	0.89	0.73	0.47	0.34	0.87
3,5,3′-Triiodothyroacetic acid	0.33	0.85	0.63	0.57	0.39	0.81
3,5-Diiodothyroacetic acid	0.28	0.87	0.64	0.58	—	0.77
3,5,3′,5′-Tetraiodothyropyruvic acid	0.21	—	0.67	0.49	—	—
3,5,3′-Triiodothyropyruvic acid	0.30	—	0.59	0.58	—	—
3,5,3′,5′-Tetraiodothyrolactic acid	0.19	—	0.61	0.43	—	—
3,5,3′-Triiodothyrolactic acid	0.27	—	0.55	0.53	—	—
3,5,3′-Triiodothyronamine	0.58	—	0.23	0.65	—	—
Iodide	0.33	0.26	0.10	0.55	0.37	3.0

TABLE II

R_F VALUES OF ACYL DERIVATIVES

Compound	*System*				
	a	*b*	*c*	*d*	*e*
N-Acetylthyroxine	0.21	0.82	0.52	0.45	—
N-Acetyl-3,5,3′-triiodothyronine	0.32	0.77	0.48	0.55	—
N-Acetyl-3,3′,5′-triiodothyronine	0.14	0.85	0.55	0.41	—
N-Acetyl-3,5-diiodothyronine	0.31	0.77	0.45	0.53	—
N-Acetyl-3,3′-diiodothyronine	0.27	0.79	0.43	0.47	—
N-Acetyl-3-monoiodotyrosine	0.13	0.72	—	—	0.21
N-Acetyl-3,5-diiodotyrosine	0.08	0.77	—	—	0.13
N-Acetyl-3-monoiodohistidine	0.11	0.33	—	—	0.15
O,N-Diacetylthyroxine	0.54	0.85	0.63	0.77	—
O,N-Diacetyl-3,5,3′-triiodothyronine	0.70	0.82	0.59	0.89	—
O,N-Diacetyl-3,3′,5′-triiodothyronine	0.40	0.86	0.63	0.67	—
O,N-Diacetyl-3,5-diiodothyronine	0.68	0.83	0.57	0.85	—
O,N-Diacetyl-3,3′-diiodothyronine	0.63	0.82	0.58	0.80	—
N-Carbobenzoxythyroxine	0.35	0.92	0.72	0.60	—
N-Carbobenzoxy-3,5,3′-triiodothyronine	0.49	0.88	0.63	0.67	—
N-Carbobenzoxy-3,3′,5′-triiodothyronine	0.32	0.92	0.75	0.57	—
N-Carbobenzoxy-3,5-diiodothyronine	0.45	0.87	0.61	0.66	—
N-Carbobenzoxy-3,3′-diiodothyronine	0.43	0.89	0.66	0.64	—
N-Carbobenzoxy-3-monoiodotyrosine	0.33	0.83	—	—	0.38
N-Carbobenzoxy-3,5-diiodotyrosine	0.26	0.89	—	—	0.30
N-Carbobenzoxy-3-monoiodohistidine	0.30	0.57	—	—	0.32
O,N-Dicarbobenzoxy-3,5-diiodotyrosine	0.66	—	—	—	0.56
O,N-Dicarbobenzoxy-thyroxine	0.65	—	0.80	0.82	—
O,N-Dicarbobenzoxy-3,5,3′-triiodothyronine	0.68	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′,5′-triiodothyronine	0.63	—	0.80	0.81	—
O,N-Dicarbobenzoxy-3,5-diiodothyronine	0.67	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′-diiodothyronine	0.67	—	0.76	0.83	—
N-Acetyl-O-methyl-thyroxine	0.33	—	—	0.57	—
N-Acetyl-O-methyl-3,5,3′-triiodothyronine	0.40	—	—	0.63	—
N-Acetyl-O-methyl-3,3′,5′-triiodothyronine	0.30	—	—	0.54	—
N-Acetyl-O-methyl-3,5-diiodothyronine	0.37	—	—	0.60	—
N-Acetyl-O-methyl-3,3′-diiodothyronine	0.35	—	—	0.58	—
O-Acetyl-tetraiodothyropyruvic acid	0.49	—	0.77	0.62	—
O-Acetyl-3,5,3′-triiodothyropyruvic acid	0.50	—	0.69	0.69	—
O-Acetyl-tetraiodothyrolactic acid	0.25	—	0.75	0.45	—
O-Acetyl-3,5,3′-triiodothyrolactic acid	0.32	—	0.32	0.54	—
O,O-Diacetyl-tetraiodothyrolactic acid	0.50	—	0.81	0.60	—
O,O-Diacetyl-3,5,3′-triiodothyrolactic acid	0.51	—	0.51	0.66	—
N-Acetyl-3,5,3′-triiodothyronamine	0.85	—	0.63	—	—
O,N-Diacetylthyroxamine	0.90	—	0.78	—	—

different solvent systems, may be reduced to negligible proportions if these criteria are supplemented by evidence of the formation and chromatographic mobility of suitably chosen derivatives. The requirements which determine the suitability of derivatives for identification purposes may be summarised thus: they should chromatograph as compact spots and be well separated from likely contaminants, they should constitute modifications of different reactive centres on the parent molecule, should be capable of formation in near quantitative yields and, where the available amounts of material are low, should be capable of being prepared in sequence. A procedure of characterising steroids by chromatography of the free compounds and of derivatives, prepared in sequence has already been described[14].

TABLE III

R_F VALUES OF METHYL ESTERS AND METHYL ETHERS

Compound	*System*		
	a	*c*	*d*
Thyroxine methyl ester	0.77	0.40	—
3,5,3′-Triiodothyronine methyl ester	0.86	0.30	—
3,3′,5′-Triiodothyronine methyl ester	0.70	0.46	—
3,5-Diiodothyronine methyl ester	0.82	0.26	—
3,3′-Diiodothyronine methyl ester	0.79	0.36	—
3-Monoiodotyrosine methyl ester	0.75	0.24	—
3,5-Diiodotyrosine methyl ester	0.57	0.27	—
3-Monoiodohistidine methyl ester	0.60	0.07	—
Tetraiodothyroacetic acid methyl ester	0.88	0.85	—
3,5,3′-Triiodothyroacetic acid methyl ester	0.91	0.79	—
3,5-Diiodothyroacetic acid methyl ester	0.90	0.78	—
Tetraiodothyropyruvic acid methyl ester	0.90	0.82	—
3,5,3′-Triiodothyropyruvic acid methyl ester	0.93	0.75	—
Tetraiodothyrolactic acid methyl ester	0.85	0.77	—
3,5,3′-Triiodothyrolactic acid methyl ester	0.90	0.72	—
O-Methyl-thyroxine	0.32	0.32	0.47
O-Methyl-3,5,3′-triiodothyronine	0.33	0.27	0.50
O-Methyl-3,5-diiodothyronine	0.31	0.28	0.49
O-Methyl-tetraiodothyroacetic acid	0.51	0.87	0.58
O-Methyl-3,5,3′-triiodothyroacetic acid	0.48	0.85	0.63
O-Methyl-3,5-diiodothyroacetic acid	0.49	0.85	0.63

Suitable derivatives for the characterisation of iodothyronines, iodotyrosines and their metabolites have been found to be methyl esters, N-acetates and O,N-diacetates, or the corresponding carbobenzoxy derivatives, and the O-methyl ethers; R_F values of these compounds in a number of different solvent systems are listed in Tables I, II and III. These derivatives are readily prepared in near quantitative yield. Characterisation of small quantities of iodothyronines and iodotyrosines, as exemplified by the identification of 3,3′,5′-triiodothyronine, a trace metabolite of thyroxine occurring in the blood plasma of plaice, is based on chromatography of the parent amino acid and on the preparation and chromatography, in sequence, of the methyl ester, O,N-diacetates, N-acetates and N-acetyl-O-methyl derivatives. Characterisation of iodothyrolactic acids is based on chromatography of the parent acids and on the preparation and chromatography of the methyl esters, O,O-diacetates and of O-acetyl derivatives having an unesterified phenolic hydroxyl group. The derivatives recommended for characterising iodothyro-pyruvic and -acetic acids are, respectively, methyl esters, O-acetates, O-methyl ethers and methyl esters, O-methyl methyl esters and O-methyl acids.

The procedures described in this communication have been in routine use in these laboratories for the last twelve months and have been applied to the analysis of iodoamino acids in a variety of biological materials including thyroid tissue, blood, urine and bile. Chromatography of the parent compounds and preparation of two derivatives have commonly proved to provide adequate criteria for identification purposes; however, when sufficient material is available, preparation of the third derivative is recommended.

ACKNOWLEDGEMENTS

The work described in this communication forms part of the programme of the Marine Laboratory, Department of Agriculture and Fisheries for Scotland. It was carried out during the tenure, by one of us (R.H.O.) of a Treasury Research Fellowship. We thank the Director, Torry Research Station (Ministry of Technology) for the temporary provision of facilities in his laboratories and Dr. ROSALIND PITT-RIVERS, Prof. H. A. LARDY, Dr. L. SCHUTTE and Dr. L. N. STARKER (Warner Lambert Research Institute) for gifts of authentic iodoamino acids.

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CHROM. 3923

A NEW ALDEHYDOGENIC PHOSPHOLIPID—AN ARTIFACT

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SUMMARY

The storing of phosphatide plasmalogens in a solution of chloroform–methanol resulted in the formation of an artifact which was easily detectable by thin-layer chromatography after reduction with lithium aluminum hydride. The reduction product reacted positively to phenylhydrazine–sulfuric acid reagent. This property, together with its chromatographic behavior on thin layers of Silica Gel G suggested the artifact as an addition product of plasmalogens and methanol. The structure of the compound (artifact) was determined by infrared and nuclear magnetic resonance spectroscopy as well as by mass spectrometry. The possible biological significance of such an addition reaction is discussed.

INTRODUCTION

Our recent analysis[1] of ethanolamine phosphatide from hog spinal cord indicated that 90% of it was in the plasmalogen form. Hence its availability as a starting material for the semisynthetic preparation of plasmalogens* (ref. 2) was obvious. However, contrary to expectation, the ethanolamine phosphatide on reduction with lithium aluminum hydride ($LiAlH_4$)[3] and subsequent chromatography (Fig. 1) yielded only small amounts of glyceryl 1-alk-1′-enyl ethers. Instead, a substantially larger amount of a more polar compound than glyceryl 1-alk-1′-enyl ether was the major component. This compound was aldehydogenic in character as indicated by its positive reaction with 2,4-dinitrophenylhydrazine–sulfuric acid reagent[4]. Surprisingly, after acetylation, the infrared spectrum of this compound (Fig. 2) failed to show any vinyl ether absorption at 6.0 μ and showed only ether absorption at 8.9 μ. In the present communication we report the isolation and characterization of this new compound and discuss its possible origin.

* The glyceryl 1-alk-1′-enyl ether obtained from ethanolamine phosphatide of hog spinal cord by $LiAlH_4$ reduction is first acetylated and then interesterified with a known fatty acid methyl ester in presence of sodium methoxide to convert it to neutral plasmalogens.

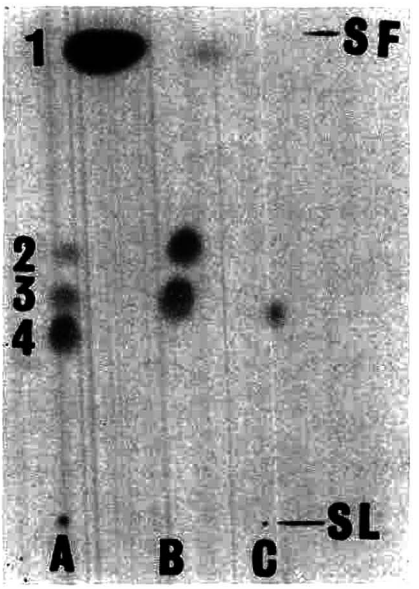


Fig. 1. Thin-layer chromatography of $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–ethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification 1 = long-chain alcohols; 2 = glyceryl 1-alk-1′-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound; Spots 2 and 4 showed positive reaction with acidic 2,4-dinitrophenylhydrazine reagent[4]. A = $LiAlH_4$ reduction products of ethanolamine phosphatide from hog spinal cord; B = standard mixture of glyceryl 1-alk-1′-enyl ether (2) and glyceryl 1-alkyl ether (3); C = standard glyceryl 1-alkyl ether (3). SL = Spotting line; SF = solvent front.

EXPERIMENTAL

Isolation of ethanolamine phosphatides from hog spinal cord

Fresh hog spinal cords (2,000 g, wet weight) collected immediately after the killing of the animals, were transported to the laboratory from the slaughter house on dry-ice. The spinal cords were immediately passed through a meat grinder a number of times, and then the entire material was freeze-dried, yielding 250 g of dry material.

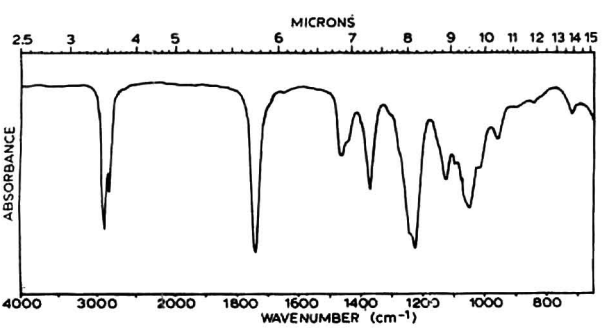


Fig. 2. Infrared analysis of the unknown compound (acetylated product).

One hundred grams of the freeze-dried material was extracted successively with a total volume of 4000 ml of a chloroform–methanol (2:1) mixture (four extractions with 1000 ml each time). The chloroform–methanol extract was then evaporated to dryness on a rotary evaporator and the residue (70 g of lipids), after dissolving in absolute chloroform, was subjected to chromatography on 400 g of acid-washed florosil column with the following dimensions: height 80 cm and internal diameter 6 cm.

The neutral lipids were first eluted with 2 l of chloroform and discarded. This was followed by further elution with chloroform–methanol mixtures (5 l) containing from 0% up to 15% of methanol. These eluates were combined and analyzed by thin-layer chromatography. The thin-layer analysis indicated a mixture containing cerebrosides and ethanolamine and serine phosphatides. The combined eluates were freed of the solvent on a rotary evaporator under suction, the residue dissolved in a minimum amount of diethyl ether and left at 0° in a cold room for 48 h. This favored the precipitation of the cerebrosides from the lipid mixture. After precipitation they were removed by cold filtration. After two successive reprecipitations, the crude ethanolamine phosphatides were rechromatographed on acid-washed florosil columns under conditions similar to those described earlier and yielded 15 g of a fairly pure fraction of ethanolamine phosphatide. This was repurified by preparative thin-layer chromatography before subjecting it to $LiAlH_4$ reduction.

Reduction of ethanolamine phosphatide with $LiAlH_4$[3]

Three grams of $LiAlH_4$ were suspended in 100 ml of absolute anhydrous diethyl ether in a three-necked, 250 ml round-bottom flask and refluxed for 30 min. This suspension was then slowly cooled to −60° in a dry-ice acetone bath, and then a solution of 5 g of ethanolamine phosphatide dissolved in 50 ml of anhydrous diethyl ether was slowly and cautiously added. After bringing the reaction mixture to room temperature, it was refluxed for 2h. The reaction mixture was then lowered to a temperature of −10° in a dry-ice acetone bath, and the unreacted excess $LiAlH_4$ destroyed by cautious addition of 7.5 ml of ethyl acetate. The reaction mixture was then transferred to a separatory funnel containing one liter of water and subsequently extracted with diethyl ether. The emulsions that resulted during the extractions were broken down by centrifugation.

After evaporating the ether extract on a rotary evaporator under vacuum, the residual lipids were redissolved in dry chloroform and then filtered through anhydrous sodium sulfate. The filtrate was then taken in a 50 ml round-bottom flask and evaporated to dryness on a rotary evaporator under vacuum, yielding 3.25 g of residue.

The residual lipids were then acetylated at room temperature overnight in a mixture of 20 ml pyridine and 10 ml acetic anhydride under continuous agitation with a magnetic stirrer. After overnight acetylation, excess of the reagents was removed mostly on a rotary evaporator under vacuum, with intermittent addition of benzene, and any residual reagents still remaining were subsequently removed under high vacuum. The acetylation products were fractionated by preparative thin-layer chromatography in the system Silica Gel G/petroleum ether–diethyl ether (85:15). The new (unknown) compound, which was observed with 2,7-dichlorofluorescein reagent (0.2% in ethanol) under ultraviolet light, was scraped off and extracted with chloroform–methanol (2:1). This extract was washed with water to remove the fluorescein and methanol. The chloroform extract, after drying over anhydrous sodium

sulfate, on removal of the solvent on a rotary evaporator yielded 1.0 g of acetylated product, which was subjected to instrumental analysis.

Thin-layer chromatography

Glass plates (20 × 20 cm) coated with Silica Gel G were air-dried for 30 min and then activated for 1 h at 110° before use.

Infrared spectroscopy

Infrared spectra were determined with a Perkin-Elmer Model 21 double beam infrared spectrometer equipped with sodium chloride optics. All the spectra were determined as liquid films.

Nuclear magnetic resonance spectroscopy

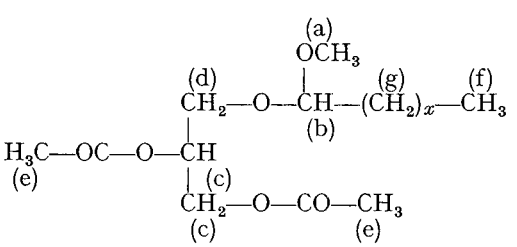
An NMR spectrum was obtained using a Varian Associate high-resolution spectrometer, Model A-60, with a probe temperature of 33°. Deuterochloroform was used as a solvent (solution concentration 10%) and tetramethylsilane was the internal standard.

Mass spectrometry

The mass spectrum was determined on a consolidated Electrodynamic Corporation Model 21-103 mass spectrometer. The experimental conditions were as follows: ionizing current, 15 μA; magnetic current, 0.56 A (for low mass) and 1.12 A (for high mass); the heated inlet, maintained at 280°, had a 3 l reservoir; ionizing voltage, 70 eV; and chamber temperature, 250°.

RESULTS AND DISCUSSION

The aldehydogenic property of the unknown compound, together with an ether absorption at 8.9 μ and *absence* of a vinyl ether absorption at 6.0 μ in the infrared spectrum of its acetyl derivative, suggested as possibilities either a cyclic acetyl or an addition product of the plasmalogen at the vinyl ether linkage[5,6]. However, the R_F-value of the acetyl derivative of the unknown compound relative to the acetyl derivative of the glyceryl 1-alk-1'-enyl ether (Fig. 3) eliminated the cyclic acetal[7] possibility. Hence the following structure seemed reasonable:



(I)

x = homologs containing 30% of saturated 14-carbon chain, 25% of saturated 16-carbon chain, and 45% monounsaturated 16-carbon chain.

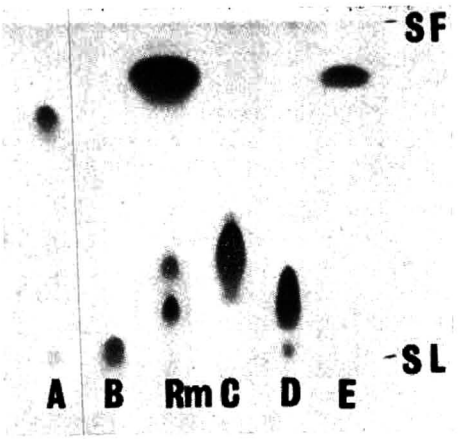


Fig. 3. Thin-layer chromatography of the unknown compound obtained by $LiAlH_4$ reduction of ethanolamine phosphatide of hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether (85:15). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification: A = tripalmitin; B = triacetin; Rm = $LiAlH_4$ reduction products of ethanolamine phosphatide of hog spinal cord after acetylation; C = mixture of glyceryl 1-alk-1'-enyl ether and glyceryl 1-alkyl ether; D = unknown compound after acetylation (isolated from Rm); E = palmityl acetate. SF = Solvent front; SL = spotting line.

This was confirmed by nuclear magnetic resonance spectroscopy and mass spectrometric analysis.

Nuclear magnetic resonance spectroscopy

For structure I the 'a' protons were observed as a sharp singlet (Fig. 4) at 3.28 p.p.m. The spectrum integral for this singlet was 10.5 divisions, *i.e.*, 3.5 divisions per proton. The same factor was used throughout in calculating the number of protons in each group.

The 'b' proton in the 5.0 to 5.5 p.p.m. region could not be clearly distinguished because of interference with the chain olefinic protons. Approximately 50% of the chains were monounsaturated. With two hydrogens per unsaturated bond, about 3.5 integral divisions should be contributed by these hydrogens to the 5.0 to 5.5 p.p.m.

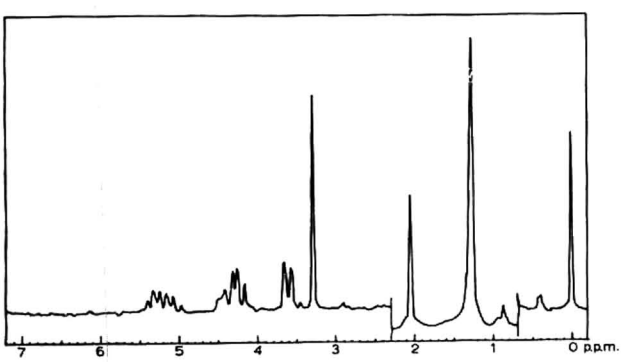


Fig. 4. Nuclear magnetic resonance spectrum of the acetylated derivative of the unknown compound.

region. Approximately 7.0 integral divisions were found experimentally, thus as expected the olefinic protons and the 'b' proton each contributed 3.5 integral divisions to the 5.0 to 5.5 p.p.m. region.

The 'c' protons were observed as a multiplet between 4.05 to 4.55 p.p.m. Even though the absorptions for these three protons were superimposed, the doublet of the $-CH_2$-group was clear. The 10.5 integral divisions found in this region gave clear indication that only three protons of the 'c' type were present in our compound.

The 'd' protons appeared as the expected doublet centering at 3.6 p.p.m. and contributed the expected 7.0 integral divisions.

The 'e' protons of the two methyls in the two acetyl groups were observed as the expected sharp singlet at 2.07 p.p.m. Approximately 21.0 integral divisions would be expected for these six hydrogens. Experimentally, however, 28 integral divisions were observed in this region. This apparent discrepancy of 7 integral divisions could be easily explained as follows: the absorptions for the four methylene hydrogens adjacent to the double bond in the chain are superimposed as a broad region under the sharp singlet. Since only about 50% of the chains were unsaturated, approximately 7 integral divisions should be contributed by these methylene protons.

The terminal methyl 'f' protons of the long chains gave a badly resolved triplet centering at 0.88 p.p.m. The 10.5 integral divisions found for these protons indicated that there was only one of these methyl groups per each molecule.

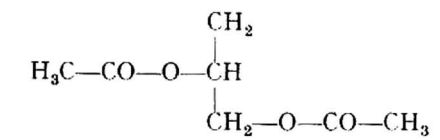
The methylene hydrogens 'g' of the aliphatic chains gave a sharp peak at 1.27 p.p.m. One hundred and four integral divisions were obtained for these protons. This was equivalent to thirty protons or around fifteen methylene groups. This was in good agreement with the proposed structure. (By gas–liquid chromatographic analysis of the aldehydes liberated from this unknown compound by acid hydrolysis, it was observed that the unknown compound contained approximately 30% hexadecanal, 25% octadecanal and 45% octadecenal.)

Mass spectrometry

In agreement with the nuclear magnetic resonance spectroscopy analysis, the mass spectrometric studies confirmed the postulated structure I.

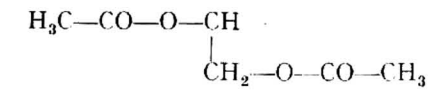
Due to lability of the molecule under electron impact, the molecular ion was not observed. However, strong fragment peaks that were characteristic of the molecule were observed at masses 159, 145, 283, 281 and 255. Using these five fragment peaks, the structure was deduced.

The strongest peak was at mass 159 due to the fragment of elemental composition $C_7H_{11}O_4$. The structure of this fragment could be:



(II)

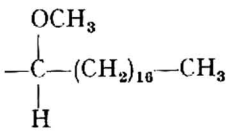
The moderately strong peak at mass 145 was due to the fragment of elemental composition $C_6H_9O_4$. The structure of this fragment could be:



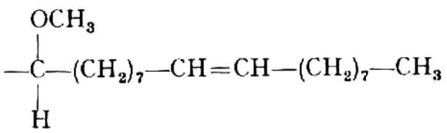
(III)

Thus, the original compound seems to be 1,2-diacetate rather than 1,3-diacetate.

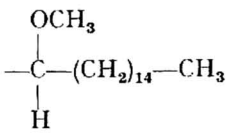
The moderately strong peaks at masses 283, 281 and 255 could be due to fragments of elemental composition $C_{19}H_{39}O$, $C_{19}H_{37}O$ and $C_{17}H_{35}O$, respectively. The structures of these fragments were rationalized as IV, V and VI, respectively:



(IV)

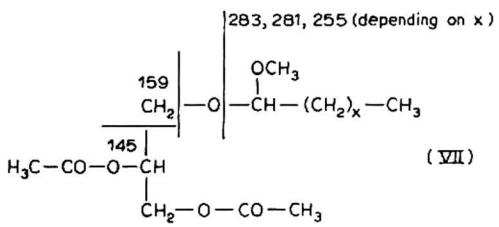


(V)



(VI)

The over-all fragmentation pattern thus appeared to be as follows:



(VII)

Elemental analysis

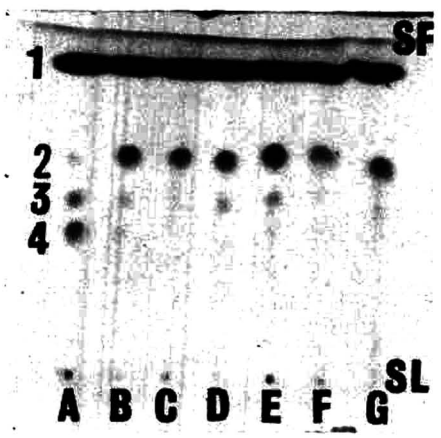
Nuclear magnetic resonance spectroscopy and mass spectrometric analysis were further substantiated by elemental analysis (Table I).

TABLE I

ELEMENTAL ANALYSIS OF THE ACETYL DERIVATIVE OF THE UNKNOWN COMPOUND

Element	*Theory*	*Obtained*
C	67.91	68.10
H	10.68	10.69
O	21.41	20.94

The formation of this type of compound in high amounts from ethanolamine phosphatides of hog spinal cord suggested investigations of their formation from other tissue phosphatides high in plasmalogens. On testing seven such samples, which also contained both choline- and ethanolamine plasmalogens, it was observed (Fig. 5)



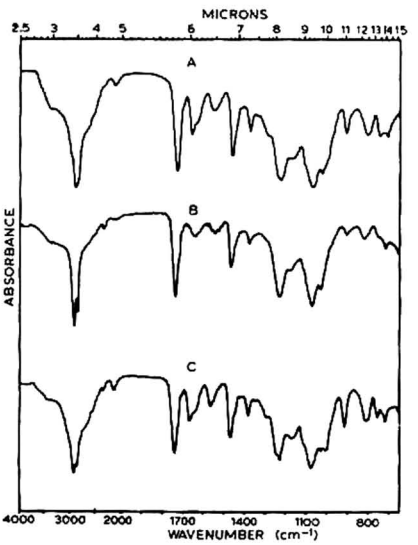


Fig. 5. Thin-layer chromatography of seven phosphatides. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification: 1 = long-chain alcohols; 2 = glyceryl 1-alk-1′-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound. Spots 2 and 4 reacted positively toward acidic 2,4-dinitrophenylhydrazine reagent. A = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog spinal cord; B = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog brain; C = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog heart; D = $LiAlH_4$ reduction product of choline phosphatide of hog heart; E = $LiAlH_4$ reduction product of ethanolamine phosphatide of beef spinal cord; F = $LiAlH_4$ reduction product of ethanolamine phosphatide of beef heart; G = $LiAlH_4$ reduction product of choline phosphatide of beef heart. SF = Solvent front; SL = spotting line.

Fig. 6. Infrared spectra of (A) ethanolamine phosphatide of beef spinal cord, (B) ethanolamine phosphatide of hog spinal cord (artifact), and (C) ethanolamine phosphatide of hog spinal cord (no artifact)

that only the ethanolamine phosphatides isolated from hog spinal cord on $LiAlH_4$ reduction could produce this unusual compound in high proportions. Did this mean that the ethanolamine phosphatides isolated from hog spinal cord differed from the ethanolamine phosphatides isolated from other tissues? This was verified by comparing the infrared spectrum of ethanolamine phosphatide isolated from hog spinal cord (Fig. 6B) with the infrared spectrum of ethanolamine phosphatide isolated from beef spinal cord (Fig. 6A). As anticipated the infrared spectrum of the ethanolamine phosphatide isolated from hog spinal cord was devoid of vinyl ether absorption, while the infrared spectrum of the ethanolamine phosphatide isolated from beef spinal cord showed a strong vinyl ether absorption, thus confirming our TLC (Fig. 5) and infrared (Fig. 2) observations. However, the absence of vinyl ether absorption in the infrared spectrum of the former compound contradicted our unpublished observation made during the analytical studies of ethanolamine phosphatides of hog spinal cord[1]. Thus it was clear that the artifact had developed during the isolation of ethanolamine phosphatide from hog spinal cord.

This isolation, as compared to that of other tissue phosphatides, alone was

carried out on a large scale for the purpose of acquiring sufficiently larger amounts of glyceryl 1-alk-1'-enyl ethers as starting material for the plasmalogen synthesis[2] and hence had remained in contact with a mixture of chloroform–methanol (2:1) for a longer period (about six weeks); supposedly this could favor development of an artifact[5,6].

To confirm this point, 100 g of fresh hog spinal cord, after grinding and freeze-drying, was immediately extracted with chloroform–methanol (2:1). The chloroform–methanol extract was concentrated and fractionated preparatively on layers of Silica Gel G with chloroform–methanol–ammonia (70:30:5) as the developing solvent, and the separated ethanolamine phosphatide isolated without any delay. The infrared analysis of this sample (Fig. 6C) showed strong vinyl ether absorption similar to that of beef spinal cord ethanolamine phosphatides (Fig. 6A), and its $LiAlH_4$ reduction product showed a complete absence of the new compound, but presence of larger amounts of glyceryl 1-alk-1'-enyl ether (Fig. 7). These observations confirmed that the procedure previously employed led to the formation of the artifact from the plasmalogens.

Compounds of similar chromatographic and infrared properties were recently reported by THOMPSON AND LEE[3] in four out of five mollusca examined after $LiAlH_4$ reduction of their lipids. However, they did not characterize these compounds. Very possibly their compounds also were artifacts.

RAPPORT[5] observed that lipids containing a high concentration of plasmalogens lost their unsaturated ether linkage (as determined by specific iodination reaction[8])

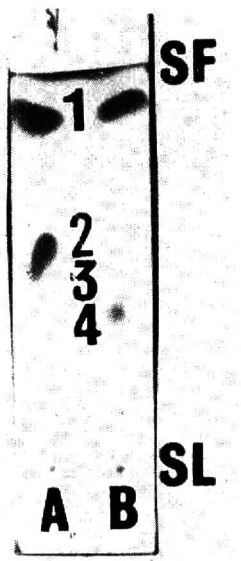


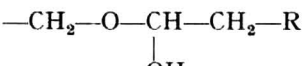
Fig. 7. Thin-layer chromatography of $LiAlH_4$ reduction product of ethanolamine phosphatides from hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spots identification: 1 = long-chain alcohols; 2 = glyceryl 1-alk-1'-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound. Spots 2 and 4 reacted positively with acidic 2,4-dinitrophenylhydrazine reagent. A = $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord (no artifact); B = $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord (artifact). SF = Solvent front; SL = spotting line.

on standing at 20–24° in a chloroform–methanol solution for several days but still fully retained their capacity to generate higher fatty aldehydes as determined by *p*-nitrophenylhydrazine formation[9]. Hence he suggested an addition reaction of the unsaturated ether producing a mixed acetal. The present report not only confirmed RAPPORT's observation, although by chromatographic technique, but also confirmed his suggestion of mixed acetal formation by I.R.- and N.M.R.-spectroscopy and by mass spectrometry.

RAPPORT[5], in the same communication, suggested that the function of plasmalogens may be related to their capacity to add water-soluble compounds to the activated double bond. THIELE[10] indicated redox equilibrium between the ester phosphatides and plasmalogens as follows:

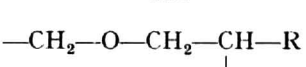
$$—CH_2—O—CO—CH_2—R + 2\,H \leftrightharpoons —CH_2—O—CH=CH—R + H_2O$$

It appears possible that an intermediate exists in this equilibrium reaction as follows:



(VIII)

or



(IX)

The former structure (VIII) may account for the abnormal compound we have observed and the latter structure (IX) may explain the occurrence of methoxy-substituted glyceryl ethers in shark liver oil[11].

Work is now in progress to label these intermediates to see whether they can be enzymatically oxidized to diester phosphatides or enzymatically dehydrated to alkenyl acyl phosphatides in various biological systems.

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CHROM. 3909

QUANTITATIVE *IN SITU* AUSWERTUNG BANDENFÖRMIG AUFGETRAGENER SUBSTANZEN IN DER DÜNNSCHICHTCHROMATOGRAPHIE

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SUMMARY

Quantitative evaluation in situ of substances applied in the form of bands in thin-layer chromatography

Quantitative evaluation *in situ* of thin-layer chromatograms following non-quantitative sample application in the form of bands has been the subject of the experiments described in this paper. The technical advantage which application in the form of bands has over application in the form of spots with respect to measurement is stressed. Non-quantitative application may be taken into consideration when relating the quantity of the components to be analysed to the quantity of a so-called internal standard. Using an example from the field of clinical analysis—the analysis of cholesterin esters of the saturated fatty acids (cholesterin stearate) in human serum—details of the procedure are described. In another part of the paper the semi-quantitative determination *in situ* of other components of a compound with reference to the components analysed quantitatively is suggested. As an example a description is given of the determination of several cholesterin esters of unsaturated fatty acids in human serum which employs the cholesterin stearate calibration curve.

In conclusion it is stated that the techniques described in the paper, *i.e.*, the application in the form of bands and the use of an internal standard for reference, do not exclude employing procedures that do not require calibration samples. Experiments in which the transferability of the extinction from one plate to another is examined, have not yet been terminated. Moreover, it should be possible to use the described method in electrophoresis without further substantial limitations.

Obwohl bei Dünnschichtchromatogrammen mit bandenförmig aufgetragenen Substanzen im Vergleich zu solchen mit punktförmiger Substanzauftragung neben einer besseren chromatographischen Trennung[1] ein geringerer photometrischer Schwierigkeitsgrad bei *in situ* Analysen zu erwarten ist, wurde seither im Routinebetrieb relativ wenig Gebrauch von diesem Verfahren gemacht. Dies erscheint darüber hinaus um so erstaunlicher, als ein Grossteil der von der Geräteindustrie seit Jahren auf den Markt gebrachten Geräte zur Photometrierung von Elektrophoresestreifen,

z.B. der Extinktionsschreiber II der Fa. Carl Zeiss, Oberkochen, sowie das Elektrophoreseauswertgerät zum Photometer Eppendorff der Fa. Netheler und Hinz, ohne weiteres für diesen Zweck eingesetzt werden kann. Die mit diesen Geräten in der Regel einstellbare rechteckige Messflächenbegrenzung ist für die hier zur Diskussion stehenden Chromatogramme im Gegensatz zu punktförmig aufgetragenen Chromatogrammen nicht nur ausreichend, sondern zwingend. Dagegen dürfte allerdings—und dies scheint ein wesentlicher Grund für die nur begrenzte Anwendung des Verfahrens zu sein—die quantitative punktförmige Auftragung der Bandentechnik überlegen sein. Im Rahmen dieser Arbeit wird aber gezeigt, dies sei an dieser Stelle vorweggenommen, dass eine quantitative bandenförmige Auftragung umgehbar ist, wenn man nur darauf achtet, dass die Auftragszonen in sich gleichmässig sind. Zur Einhaltung dieser Bedingung sind neuerdings in der Literatur eine grosse Anzahl brauchbarer Verfahren[2-4] und Geräte* angeboten worden. Hingewiesen sei an dieser Stelle auch auf die Einführung sogenannter Fertigplatten[1], die sich nicht nur durch eine für die Bandenauftragung günstig auswirkende Abriebfestigkeit auszeichnen.

Besonders zu erwähnen ist eine Veröffentlichung von ACKERMANN UND ASSMUS[5]. In dieser sich mit der Auswertung von Papierchromatogrammen beschäftigenden Arbeit wird neben einer prinzipiell auch für die Dünnschichtchromatographie anwendbaren Methode zur Herstellung gleichmässig belegter Zonen über Fehlerquellen, die bei den verschiedenen diskutierten Messverfahren auftreten können, berichtet. Unter anderem wird eine gewisse Abhängigkeit der Messwerte von der Fleckbelegung angegeben.

Diese von STAHL UND JORK und KLAUS für punktförmig aufgetragene Dünnschichtchromatogramme an anderer Stelle[6-8] ebenfalls behandelten systematischen Messwertbeeinflussungen reduzieren sich bei bandenförmiger Auftragung erheblich. So führen bei Anwendung des Durchlichtverfahrens im wesentlichen Falschlichtanteile zu einer Beeinträchtigung der Messwerte. Aufgrund dieser verminderten Messwertbeeinflussung wird in einem späteren Abschnitt auf die Möglichkeit hingewiesen, unter gewissen Voraussetzungen halbquantitative Aussagen auch von solchen Komponenten der Analysensubstanz zu machen, die nicht direkt geeicht werden.

Bezüglich der eigentlichen Analysendurchführung wurden in Anbetracht der in der Industrie bzw. in der Klinik noch in überwiegender Zahl verwendeten Geräte, die mit durchfallendem Licht arbeiten, eine Messanordnung benutzt, die diesen Gegebenheiten entspricht. Sie setzte sich aus Teilen eines Spektralphotometers PMQ II, der Firma Carl Zeiss, Oberkochen, einem Transmission-Extinktions-Wandler, einem Kompensationsschreiber und einem Chromatogrammzusatz (Eigenbau)[7] zusammen. Als Messgrössen, für deren Registrierung eine Spaltblende 6 × 0.2 mm verwendet wurde, boten sich somit die Extinktionen bzw. Extinktionsortsintegrale an.

QUANTITATIVE AUSWERTUNG BEI NICHTQUANTITATIVER BANDENFÖRMIGER AUFTRAGUNG

Wie einleitend bereits angedeutet, dürfte eine der Schwierigkeiten bei der *in situ* Analyse bandenförmiger Chromatogramme in dem quantitativen Auftragen der Substanzen auf die Dünnschicht-Platte zu suchen sein. Wir haben zur Verminderung der hierdurch bedingten Fehlermöglichkeiten daher versucht, ein auf anderen Gebieten

* z.B. Linomat, Hersteller Fa. CAMAG, Muttenz (Schweiz).

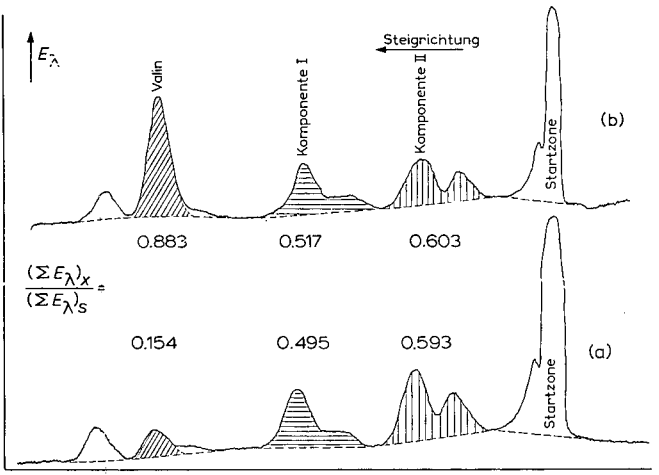

Fig. 1. Extinktionsortskurven eines dünnschichtchromatographisch getrennten Serums. (a): Normalserum; (b): Normalserum + Valin.

der Analytik mit gutem Erfolg angewandtes Verfahren auf diesen Sektor der Messtechnik zu übertragen. Bei dieser Methode des sogenannten inneren Standards wird die Messgrösse der zu analysierenden Komponente auf die Messgrösse einer konstanten Komponente bezogen. Letztere kann sowohl Bestandteil der Analysensubstanz als auch eine zu der Analysensubstanz zugesetzte Komponente sein. Von diesem so errechneten Messgrössenquotienten sollte man erwarten, dass er unabhängig von den absoluten Beträgen der beiden Messgrössen und somit von dem Auftragsvolumen ist. Voraussetzung hierfür ist allerdings, dass der funktionelle Zusammenhang zwischen den beiden Messgrössen und den dazu gehörigen Konzentrationen der Komponenten einander entsprechen, was sich einerseits durch eine sinnvolle Wahl der Bezugskomponente, andererseits durch eine entsprechende Verdünnungsstufe der Analysenlösung fast immer erreichen lässt.

Diese theoretische Überlegung der Konstanz der Quotienten wurde an einer Reihe von Dünnschichtchromatogrammen überprüft. Von einem solchen zeigt Fig. 1 die Extinktionsortskurven zweier Bahnen des gleichen Serums bei verschiedenen aufgetragenen Mengen. Die Darstellung bzw. die Auswertung der Kurven bestätigt den erwarteten Sachverhalt. Die Quotienten der integrierten Extinktionen

$$\frac{(\Sigma E_\lambda)\ \text{Komp. I bzw. II}}{(\Sigma E_\lambda)\ \text{Startzone}}$$

ergeben einen annähernd konstanten Wert. Gleichzeitig gibt Fig. 1 ebenso wie die folgende Fig. 2 die Veränderung der Werte

$$\frac{(\Sigma E_\lambda)x}{(\Sigma E_\lambda)\ \text{Startzone}}$$

bei qualitativer Zugabe verschiedener Komponenten zu dem Serum wieder.

Darüber hinaus ist aus den Extinktionsortskurven von Fig. 2 zu ersehen, dass trotz der hier gewählten optimalen Bedingungen bei der chromatographischen Vorbereitung der Platte nur eine ungenügende Trennung der einzelnen Komponenten

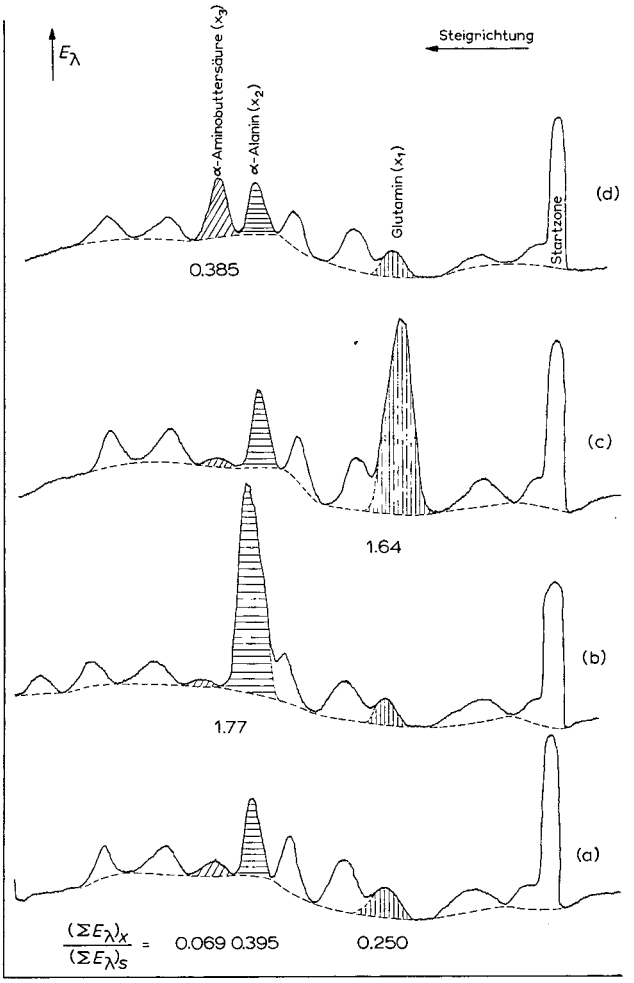

Fig. 2. Extinktionsortskurven eines dünnschichtchromatographisch getrennten Serums. (a): Normalserum; (b): Normalserum + α-Alanin; (c): Normalserum + Glutamin; (d): Normalserum + α-Aminobuttersäure.

erzielt wurde, so dass eine graphische Korrektur der registrierten Banden erforderlich ist. Auf diese Notwendigkeit sei hingewiesen, da sich auch bei optimal gewählten Verhältnissen von Spaltbreite zu Bandenbreite dieser Effekt häufig nicht vermeiden lässt.

Diese Gesichtspunkte seien der eigentlichen Beschreibung der quantitativen Analyse, für die sich im wesentlichen die drei folgenden Verfahren anbieten, vorausgestellt:

(a) Die Analyse erfolgt unter Anwendung von Eichkurven, die mit Reinsubstanzen der zu prüfenden Komponenten bzw. des zugegebenen inneren Standards erstellt werden.

(b) Die Analyse wird unter Verwendung von Eichsubstanzen durchgeführt, welche sich aus sämtlichen Komponenten chromatographiereiner Qualität der zu prüfenden Substanz zusammensetzen. Sind in der Analysensubstanz Komponenten

in geringerer Konzentration zu bestimmen, so bietet sich eine Nebenkomponente als innerer Standard an.

(c) Die zu prüfende Substanz wird nach dem Zumischverfahren mit nachfolgender Extrapolation analysiert.

Während das Verfahren (b) unter anderem aus Beschaffungsgründen der reinen Grundsubstanzen nur einen begrenzten Anwendungsbereich haben dürfte, das Verfahren (a) aber z.B. bei Spurenanalysen aufgrund der für Proben- und Vergleichsbahn verschiedenartigen chromatographischen Bedingungen u.U. Korrekturverfahren erfordern würde, legten wir unseren Analysen die Methode (c) zugrunde.

Das Zumischverfahren geht von der Voraussetzung aus, dass die zu der zu analysierenden Substanz zugesetzten Mengen der zu bestimmenden Komponente den gleichen Beeinflussungen unterliegen wie der ursprünglich vorhandene Anteil selbst. Auf diese Notwendigkeit sei hingewiesen, da das Verfahren bei Nichterfüllung dieser Bedingung an Richtigkeit verlieren kann. Darüber hinaus sollten die Analysenbedingungen so gewählt werden, dass man den linearen bzw. annähernd linearen Bereich der Messwert-Konzentrationskurve erreicht. Dies lässt sich in der Regel durch eine entsprechende Verdünnung der Analysenlösung bzw. durch die Wahl der Zusätze herbeiführen.

Mit einem Beispiel aus dem Bereich der Serumanalytik soll das Verfahren vorgeführt werden. Der eigentliche chromatographische Trennungsgang, der zur Isolierung der zu bestimmenden Cholesterinester der gesättigten Fettsäuren (Cholesterinstearat) führt, sei als gegeben vorausgesetzt und lediglich in der Legende zu Fig. 3 kurz angedeutet. Auf die Herstellung der aufgetragenen Lösungen soll im folgenden näher hingewiesen werden, da sie der Gehaltsberechnung zugrunde gelegt werden

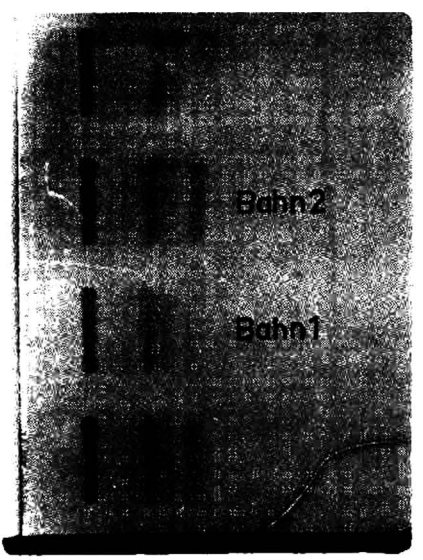

Fig. 3. Photographisches Positiv der für die Identifizierung von Cholesterinstearat in Serum hergestellten Platte. Bahn 1: Normalserum. Bahn 2: Normalserum + Cholesterinstearat (5 mg/10 ml). Sorptionsmittel: DC-Fertigplatte Kieselgel F_{254}, 20 × 20 cm. Fliessmittel: Petroleumbenzin (60–80°)–Chloroform (90:10), viermalige Entwicklung je 10 cm hoch. Nachweis: Besprühen mit 20%iger Lösung von Molybdatophosphorsäure in Äthanol, anschliessend etwa 2 Min. auf 120° erwärmen.

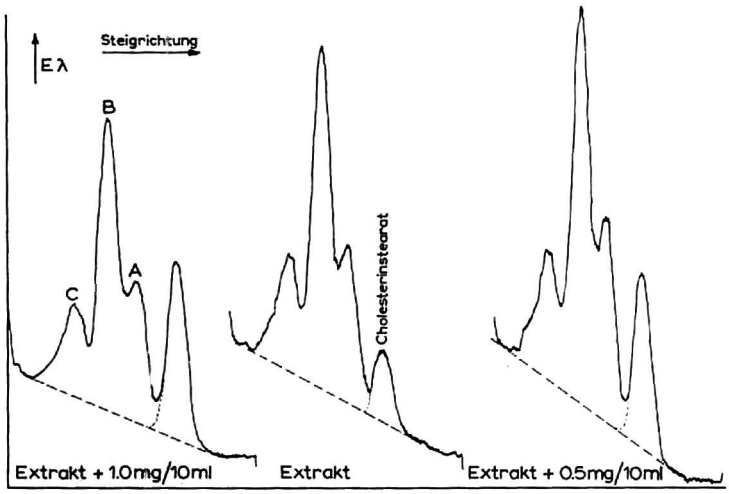

Fig. 4. Extinktionsortskurven der für die quantitative Analyse von Cholesterinstearat hergestellten Platte.

müssen. Als Ausgangslösung wurde eine Lösung des Extraktes von 2.5 ml Normal-Serum in 20 ml Chloroform gewählt. Nachdem in einem Vorchromatogramm— Fig. 3 zeigt ein photographisches Positiv eines Plattenausschnittes— der R_F-Wert der Analysenkomponente ermittelt war, wurden zu Teilen der Ausgangslösung 0.5 mg und 1.0 mg Cholesterinstearat, berechnet auf 10 ml Ausgangslösung, zugesetzt. Von diesen so erstellten Lösungen wurden aliquote Teile mit einer Blutzuckerpipette bandenförmig aufgetragen und anschliessend chromatographiert. Schliesslich wurden die Extinktionsortskurven der einzelnen Bahnen der Dünnschicht-Platte mit λ = 600 nm (Fig. 4) registriert und integriert (Tabelle I). Als innerer Standard bietet sich bei diesem Beispiel die Summe der drei vor dem Analysenpeak liegenden Komponenten an. Trägt man nun die in Tabelle I für die verschiedenen Bahnen errechneten Quotien-

TABELLE I

WERTETABELLE ZUR BESTIMMUNG VON CHOLESTERINSTEARAT IN SERUM NACH DEM EXTRAPOLATIONSVERFAHREN

Bahn	$(\Sigma E_\lambda)_{Cholesterinstearat}$ *(rel. Einh.)*	$(\Sigma E_\lambda)_{innerer\ Standard}$ *(rel. Einh.)*	$\frac{(\Sigma E_\lambda)_{Cholesterinstearat}}{(\Sigma E_\lambda)_{innerer\ Standard}}$
1 Serum + 0.5 mg Cholesterinstearat (10 ml)	35.1	146.5	0.240
2 Serum	15.8	151.2	0.105
3 Serum + 1.0 mg Cholesterinstearat (10 ml)	55.9	138.3	0.403
4 wie 2	22.4	182.1	0.123
5 wie 1	44.1	183.4	0.240
6 wie 2	20.9	167.0	0.125
7 wie 3	46.8	142.2	0.329
Mittel 2,4,6			0.117
Mittel 1.5			0.240
Mittel 3.7			0.366

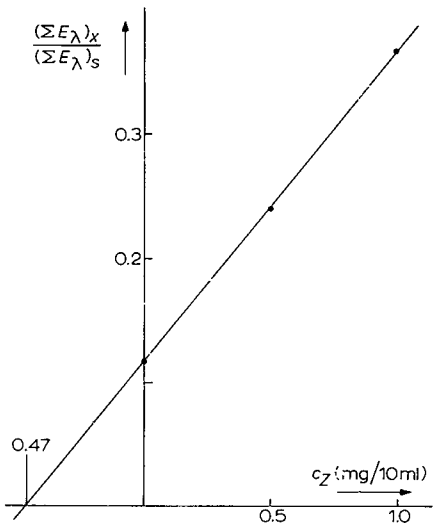

Fig. 5. Aus Tabelle I resultierende Eichkurve.

ten der integrierten Extinktionen in Abhängigkeit zu den der Ausgangslösung zugesetzten Cholesterinstearatmengen in einem Koordinatensystem auf (Fig. 5), so resultiert aus der graphischen Extrapolation der Cholesterinstearatgehalt der Ausgangslösung (0.47 mg/10 ml). Für das gewählte Serum ergab sich nach Umrechnung entsprechend der vorgenommenen Verdünnung ein Gehalt an Cholesterinestern der gesättigten Fettsäuren (Cholesterinstearat) von 3.7 mg/10 ml.

HALBQUANTITATIVE BESTIMMUNG NICHT DIREKT GEEICHTER KOMPONENTEN DER ANALYSENSUBSTANZ

In dem folgenden Abschnitt soll versucht werden, unter Anwendung der für einen Bestandteil der Analysensubstanz aufgenommenen Eichkurve, eine halbquantitative Abschätzung weiterer vorhandener, der Eichsubstanz ähnlicher Komponenten vorzunehmen. Der halbquantitative Charakter dieses Verfahrens ist im wesentlichen durch die beiden folgenden Punkte bedingt:

(a) Mögliche Abweichung der spezifischen Extinktion zwischen Eichkomponente und Analysenkomponente.

(b) Durch abweichende R_F-Werte für Eich- und Analysensubstanz hervorgerufene verschiedene Fleckbelegung und damit veränderter Falschlichteinfluss.

Unter Bezugnahme auf die Eichkurve von Cholesterinstearat werden die weiteren in Fig. 4 mit A, B und C bezeichneten Komponenten des im vorausgegangenen Abschnitt verwendeten Serums analysiert. Eine Identifizierung der zwischen der Startzone und Cholesterinstearat liegenden Bestandteile ergab, dass es sich bei diesen um die Cholesterinester der einfach, der zweifach und der dreifach ungesättigten Fettsäuren handelt. Zur halbquantitativen Auswertung werden nun die einzelnen Peakflächen der Extinktionsortskurven (Fig. 4) der drei ungesättigten Ester analog dem quantitativen Beispiel auf die Summe dieser drei Peakflächen als innerer Standard

TABELLE II

WERTETABELLE ZUR HALBQUANTITATIVEN AUSWERTUNG UNGESÄTTIGTER FETTSÄUREESTER UNTER ANWENDUNG DER EICHKURVE FÜR CHOLESTERINSTEARAT

Bahn	*Komponente*	(ΣE_λ)	$\frac{(\Sigma E_\lambda)}{(\Sigma E_\lambda)_{A+B+C}}$	*Konzentration (mg/10 ml Serum)*
2	A (einfach ungesätt. Ester)	36.8	0.232	7.4
2	B (zweifach ungesätt. Ester)	81.2	0.512	16.3
2	C (dreifach ungesätt. Ester)	34.9	0.220	7.0
3	A	36.7	0.242	7.8
3	B	85.3	0.563	18.0
3	C	30.6	0.202	6.5
4	A	54.5	0.273	8.7
4	B	107.2	0.536	17.1
4	C	38.0	0.190	6.1

bezogen. Die so erhaltenen Quotienten dienen als Masszahlen, die es erlauben, aus der Eichkurve Fig. 5 die Gehalte der einzelnen Komponenten zu ermitteln. Tabelle II gibt Aufschluss über die Werte, die aus drei Bahnen der für die Cholesterinstearatanalyse hergestellten Platte resultieren. Eine Mittelbildung führt schliesslich für das eingesetzte Serum mit den folgenden Gehalten der drei Komponenten:

A. (einfach ungesättigter Fettsäureester), 8.0 mg/10 ml Serum
B. (zweifach ungesättigter Fettsäureester), 17.1 mg/10 ml Serum
C. (dreifach ungesättigter Fettsäureester), 6.5 mg/10 ml Serum

zu Werten, deren Summe an der oberen Grenze des physiologischen Normalbereiches liegt.

ZUSAMMENFASSUNG

Die quantitative *in situ* Auswertung von Dünnschichtchromatogrammen bei nicht-quantitativer bandenförmiger Auftragung ist Gegenstand der vorstehenden Ausführungen. Insbesondere wird auf den messtechnischen Vorteil der *in situ* Auswertung bandenförmiger Chromatogramme gegenüber punktförmigen Chromatogrammen hingewiesen. Die nicht-quantitative Auftragung kann durch den Bezug der Messgrösse der Analysenkomponente auf die Messgrösse eines sogenannten inneren Standards berücksichtigt werden. Mit einem Beispiel aus dem Bereich der klinischen Analytik—einer Analyse von Cholesterinestern der gesättigten Fettsäuren (Cholesterinstearat) in Humanserum—werden Einzelheiten des Verfahrens mitgeteilt. In einem weiteren Abschnitt wird auf die Möglichkeit einer halbquantitativen *in situ* Bestimmung anderer Analysensubstanzbestandteile unter Bezugnahme auf die quantitativ analysierte Komponente hingewiesen. Als Beispiel wird die Bestimmung mehrerer Cholesterinester ungesättigter Fettsäuren in Humanserum bei Anwendung der Cholesterinstearat-Eichkurve beschrieben.

Abschliessend sei vermerkt, dass das hier erläuterte Verfahren des bandenförmigen Auftragens bzw. des Bezugs auf einen inneren Standard ein eichprobenfreies

Arbeiten nicht ausschliesst. Versuche, welche die Übertragbarkeit der Extinktionsquotienten von Platte zu Platte klären sollen, sind noch nicht abgeschlossen. Ebenso dürfte die Anwendbarkeit des Verfahrens bei der Elektrophorese ohne wesentliche zusätzliche Einschränkungen möglich sein.

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DIREKTE QUANTITATIVE BESTIMMUNG VON SCILLAGLYKOSIDEN AUF DÜNNSCHICHTCHROMATOGRAMMEN DURCH REMISSIONSMESSUNG

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SUMMARY

Direct quantitative determination of Scilla glycosides on thin-layer chromatograms by means of remission measurements

Different methods of direct quantitative evaluation of thin-layer chromatograms for the determination of bufadienolides were examined. The evaluation of remission measurements yielded errors of $s_{rel} < \pm 5\%$ for all determinations. Therefore this method seemed to be superior to transmission measurements of eluates, not only because it is faster, but also because it is more precise.

EINLEITUNG

Vor kurzem berichteten wir über eine verbesserte Arbeitsweise zur quantitativen Bestimmung von Scillaglykosiden in Extrakten und anderen Zubereitungen der Meerzwiebel durch spektralphotometrische Transmissionsmessung nach dünnschichtchromatographischer Trennung[1,2]. Für Serienanalysen war der Zeitaufwand jedoch noch immer erheblich. Wir versuchten daher, die entwickelten Chromatogramme direkt auszuwerten, um die Zeitdauer der Analysen weiter zu verkürzen. Durch vergleichende Untersuchung verschiedener Messverfahren sollte geprüft werden, welche Methode sich für die quantitative Erfassung von Bufadienoliden am geeignetsten erweist.

DENSITOMETRIE

Die densitometrische Auswertung von Dünnschichtchromatogrammen wurde in den vergangenen Jahren von verschiedenen Autoren intensiv in zwei Richtungen bearbeitet: als Transmissionsmessung[5,6] und als Reflexionsmessung[7]. Die Transmissionsmessung schied bei unseren Arbeiten aus, weil das vorgesehene Adsorbens Kieselgel mit einfachen Mitteln nicht transparent zu machen war. Ausserdem hatten INGLE UND MINSHALL[8,9] festgestellt, dass die Messungen bei reflektiertem Licht günstiger sind als bei transmittiertem Licht. Mit der reflexionsdensitometrischen

Technik auf Dünnschichtplatten wurden die verschiedensten Naturstoffgruppen wie Harze und Balsame[10], Alkaloide[11–13] und Steroide[14–16] dünnschichtchromatographisch untersucht. Ausserdem wurde über lebensmittelchemische[17] und biochemische[18] Untersuchungen berichtet. Speziell für die Densitometrie wurden verschiedene Geräte entwickelt, wie z.B. das Elphor-[19], das ERI 10-[11], das Eppendorf-[14], das Beckman-Spinco-Analytrol-[20] und das Chromoscan-Gerät[10,13,17,21,22].

Die zum Teil beachtlichen Erfolge, welche in bestimmten Fällen mit reflexionsdensitometrischen Messungen erzielt werden konnten, können jedoch nicht über die schon von WIEME[23] beschriebenen Schwierigkeiten hinwegtäuschen, welche dadurch gegeben sind, dass die entwickelten Flecken eines Chromatogrammes häufig ungleichförmig sind. Eigene Untersuchungen mit dem Chromoscan-Gerät bei der densitometrischen Auswertung der Dünnschichtchromatogramme von Mischungen aus Scillaglykosiden und Scillaextrakten ergaben für die Mischungen der Glykoside einen relativen Fehler von etwa 10% und für die Scillaextrakte von 15%. Bei Chromatogrammen von Scillarohextrakten wuchs der Fehler bis zu 20%. Dieser hohe Messfehler ist nicht zuletzt dadurch bedingt, dass die Detektionsmittel, z.B. Antimontrichlorid, häufig nicht zu stabilen Umsetzungsprodukten führen und auch der Reagenzüberschuss u.U. die Messung stört.

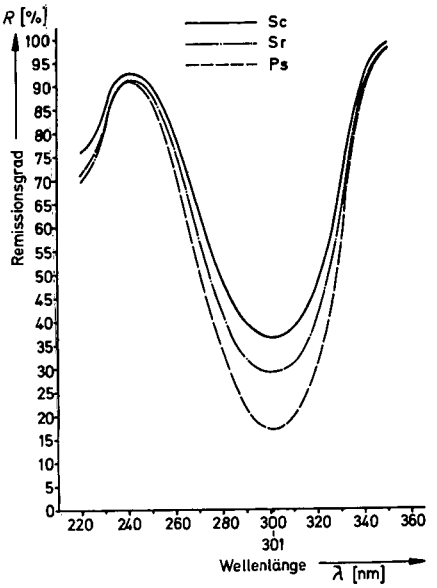
FLUOROMETRIE

Eine weitere Möglichkeit zur direkten Auswertung von Chromatogrammen bietet die Fluorometrie. Bereits 1961 berichteten WELLS *et al.*[24] über fluorometrische Bestimmungen von cardiotonischen Steroiden. KNY[25–27] bestimmte mit einem Fluorometer der Firma VEB Zeiss Digitalis-Glykoside am Papierchromatogramm. Mit einem Trichloressigsäure–Chloramin-Reagenz erhielt er gute Fluoreszenswerte. SEILER *et al.*[28] führten quantitative Bestimmungen von fluoreszierenden Substanzen auf Dünnschichtchromatogrammen mit einem Zusatzgerät zum Zeiss-Spektralphotometer PMQ II durch. KLAUS[29] setzte sich 1964 kritisch mit den Möglichkeiten der Auswertung fluorometrischer Messungen von Dünnschichtchromatogrammen auseinander und wies auf die infolge der oft beträchtlichen Abweichungen der Fleckenformen und -grössen gegebenen Grenzen dieses Messverfahrens hin, welche KNY[25] durch mathematisch statistische Überlegungen zu erweitern versucht. Fast gleichzeitig mit der Spektrophotofluorometrie wurde für die Analyse aromatischer Verbindungen auf Dünnschichtchromatogrammen eine Spektrophotophosphorimetrie entwickelt[30]. Für eigene Versuchsbestimmungen wurde das von mehreren Autoren[31–34] beschriebene Turner-Fluorometer herangezogen. Da Scillaglykoside im U.V.-Licht keine Fluoreszenz aufweisen und sich beim Besprühen der Chromatogramme zur Fluoreszenzbildung mit dem Trichloressigsäure–Chloramin-Reagenz nach KAISER[35] sich dieselben, schon bei der Densitometrie geschilderten Schwierigkeiten ergaben, konnte nur die Fluoreszenzlöschung auf Dünnschichten mit Fluoreszensindikator gemessen werden. Hierbei ergab sich bei der Auswertung der Messungen von Mischungen aus Scillareinglykosiden ein relativer Fehler von etwa 10% und bei der Auswertung von vorgereinigten Scillaextrakten ein solcher von etwa 15%. Die Chromatogramme von Scillarohextrakten auf fluoreszierenden Dünnschichten konnten mit dieser Methode überhaupt nicht ausgewertet werden, da die Begleitstoffe die Messungen störten.

U.V.-REMISSIONSMESSUNGEN

In den letzten Jahren wurden von verschiedenen Autoren Untersuchungen darüber angestellt, wie man durch die Funktion nach KUBELKA und MUNK[36,37] mit Hilfe von Remissionsmessungen Dünnschichtchromatogramme direkt quantitativ auswerten könnte. Als Grundlage dafür dienten auch Arbeiten von KORTÜM, SCHREYER u.a.[38–41] über Reflexionsspektren von Pulvern. Diese Autoren untersuchten u.a. auch die Abhängigkeit der Kubelka–Munk-Funktion von der Korngrösse des reflektierenden Pulvers. Während KORTÜM UND VOGEL[42] diese Erkenntnisse zunächst für eine quantitative Auswertung von Papierchromatogrammen durch densitometrische Reflexionsmessungen zu verwenden versuchen, ist es bei der weiteren Entwicklung dieser Messmethode vor allem die Gruppe um FRODYMA[43–49], die sich zunächt densitometrisch, später fluorometrisch und mit U.V.-Remissionsmessungen um die quantitative Auswertung von Dünnschichtchromatogrammen bemüht. Mit dem von diesen Autoren angewandten Verfahren können zwar relativ gute Messwerte erreicht werden, jedoch ist damit der Nachteil verknüpft, dass die einzelnen Flecken des Chromatogrammes zur quantitativen Auswertung von der Dünnschichtplatte abgeschabt und homogenisiert werden müssen. Erst dann wird die Substanz in einer Zelle gegen einen Bezugsstandard, z.B. MgO, gemessen. Diese Methode erfordert immer noch zeitraubende Operationen und bedeutet keine wesentliche Verkürzung der Analysendauer gegenüber Transmissionsmessungen nach Eluierung der Substanz. JORK[50] berichtete 1964 erstmals über ein Chromatogramm-Spektralphotometer der Firma Zeiss für die Dünnschichtchromatographie. In weiteren Arbeiten[4,51,52] untersuchte er, wie auch KLAUS[53], die sich aus diesem Gerät ergebenden Möglichkeiten, die vor allem darin bestehen, dass die Chromatogramme nach der Entwicklung ohne weitere Vorbereitungen direkt ausgewertet werden können. Das ist dadurch möglich, dass das Chromatogramm mit monochromatischem Licht unter einem Winkel von 0^c (bezogen auf die Flächennormale) angestrahlt und das diffus zurückgestrahlte, nicht absorbierte Licht unter einem Winkel von 45° gemessen wird. Dabei kann die Dünnschichtplatte zur Aufsuchung und Zentrierung der Substanzflecken auf einem Kreuztisch in x- und y-Richtung bewegt werden. Da diese Messmethode eine beträchtliche Zeitersparnis bringt, schien sie für die hier besprochenen Untersuchungen am besten geeignet zu sein.

Zunächst wurden die in Scillapräparaten hauptsächlich vorkommenden Scillaglykoside Scillaren A, Proscillaridin und Scillirosid dünnschichtchromatographisch getrennt und ihre U.V.-Remissionsspektren aufgenommen. Wie schon früher erwähnt[1], waren bei selbst hergestellten Dünnschichtplatten im Gegensatz zu den Berichten anderer Autoren[49] Schichtdicke und damit die Schichtstruktur nicht reproduzierbar. Da aber nicht nur die R_F-Werte[54,55], sondern auch die Remissionsbedingungen von den verschiedenen Platteneigenschaften abhängen[41,56], wurden für alle Bestimmungen Dünnschichtchromatographie-Fertigplatten Kieselgel "Merck"[57] verwendet. Ausserdem wurde auf Grund früherer Erfahrungen[1] und den Berichten anderer Autoren[58–61] die Entwicklung der Dünnschichtplatten vor allem hinsichtlich der Kammersättigung, der Temperatur und der Wasserdampfisotherme so reproduzierbar wie möglich gestaltet, indem man in einem Raum bei einer konstanten Temperatur von 25° und einer nahezu völlig konstanten relativen Luftfeuchtigkeit von 30% arbeitete. Die Chromatogramme wurden mit der von GÖRLICH[62] beschriebenen Mischung Aethylmethylketon–Toluol–Wasser–Methanol–Eisessig (80:10:6:5:2) entwickelt.



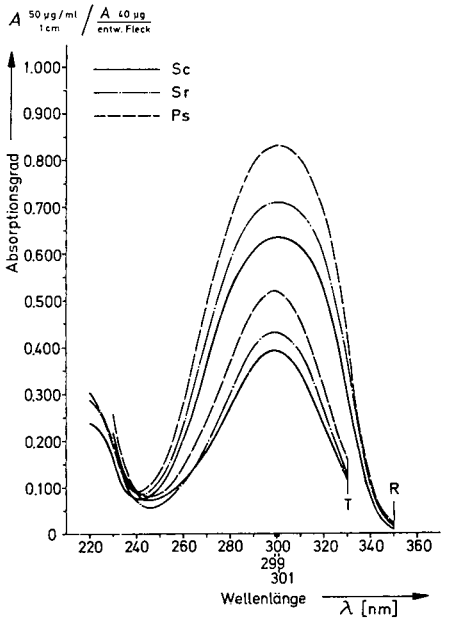


Fig. 1. Remissionsspektren einiger Bufadienolide. Sc = Scillaren A; Sr = Scillirosid; Ps = Proscillaridin.

Fig. 2. Absorptionsspektren einiger Bufadienolide bei Transmissionsmessung (T) und Remissionsmessung (R). Sc = Scillaren A; Sr = Scillirosid; Ps = Proscillaridin.

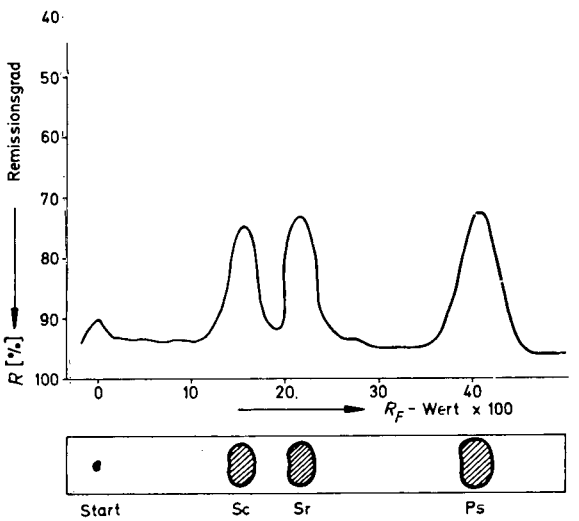


Fig. 3. Remissionsortskurve eines Mischchromatogrammes. Sc = Scillaren A; Sr = Scillirosid; Ps = Proscillaridin.

Nach der Entwicklung wurden die Dünnschichtplatten getrocknet und dann mit dem Chromatogramm-Spektralphotometer die Remissionsspektren der drei Glykoside aufgenommen (Fig. 1).

Sie wurden mit den Absorptionsspektren von Transmissionsmessungen verglichen (Fig. 2).

Wie bei anderen Substanzen[63,64] ist auch bei den Scillaglykosiden eine, wenn auch geringe Verschiebung des Absorptionsmaximums sowie eine Verbreiterung der Banden zu beobachten. Diese Erscheinung ist wahrscheinlich durch die von KORTÜM u.a.[63,65–68] untersuchte Eigenart der molekularen physikalischen Adsorptionsbindung der Substanz an das Sorbens zu erklären. Für die drei untersuchten Herzglykoside Scillaren A, Proscillaridin und Scillirosid wurde bei den Remissionsmessungen ein gemeinsames Absorptionsmaximum bei einer Wellenlänge von 301 nm gefunden.

Fig. 3 zeigt die bei dieser Wellenlänge registrierte Remissionsortskurve eines entwickelten Mischchromatogrammes der drei Glykoside.

Es ist beachtlich, dass diese Aufnahme mit nur je 4 μg der einzelnen Glykoside gelang. Die quantitative Erfassung wurde sowohl als Flächenberechnung der Halb-

TABELLE I

SCILLAREN A: VERGLEICHENDE UNTERSUCHUNGEN DER BESTIMMUNG DES GLYKOSIDES DURCH TRANSMISSIONS- UND REMISSIONSMESSUNGEN

Auftrag am Startfleck (μg)	*Transmissionsmessung des Eluates*		*Remissionsmessung der Dünnschichtplatten*			
			Fläche $B_h \cdot H$		*Fläche planimetriert*	
	Verlust (%)	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*
2.00	4.5	11.3	2.5	4.2	2.5	3.6
4.00	3.7	10.1	2.5	3.6	2.2	3.2
6.00	3.3	8.5	2.0	3.2	1.7	3.1
8.00	2.3	8.3	1.2	3.1	1.2	2.9
10.00	2.1	6.2	1.3	2.9	1.2	2.8

TABELLE II

SCILLIROSID: VERGLEICHENDE UNTERSUCHUNGEN DER BESTIMMUNG DES GLYKOSIDES DURCH TRANSMISSIONS- UND REMISSIONSMESSUNGEN

Auftrag am Startfleck (μg)	*Transmissionsmessung des Eluates*		*Remissionsmessung der Dünnschichtplatten*			
			Fläche $B_h \cdot H$		*Fläche planimetriert*	
	Verlust (%)	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*
2.00	4.4	10.8	2.4	4.4	2.5	3.7
4.00	3.7	10.0	2.4	3.7	2.3	3.2
6.00	3.3	9.6	2.3	3.2	1.8	3.0
8.00	2.2	8.3	1.5	3.2	1.3	3.1
10.00	2.1	5.9	1.3	3.1	1.3	2.7

wertsbreite B_h und der Höhe H als auch durch Planimetrieren der von der Ortskurve umschriebenen Fläche vorgenommen. Der Absorptionswert des Untergrundes wurde jeweils berücksichtigt. Die ermittelten Flächen wurden zur Auswertung quadriert.

Zum Vergleich der Messgenauigkeit des Transmissions- und Remissionsverfahrens wurden von verschiedenen Substanzkonzentrationen je neun Messungen an entwickelten Mischchromatogrammen sowohl durch direkte Remissionsmessung von der Dünnschichtplatte als auch durch Transmissionsmessung von Eluaten der Substanzflecke durchgeführt (Tabellen I–III).

In den Tabellen I bis III ist der Substanzverlust durch die chromatographische Entwicklung, d.h. die Differenz zwischen dem Mittelwert aus mehreren Messungen und dem aufgetragenen theoretischen Wert, und der Messfehler, d.h. die Streuung

TABELLE III

PROSCILLARIDIN: VERGLEICHENDE UNTERSUCHUNGEN DER BESTIMMUNG DES GLYKOSIDES DURCH TRANSMISSIONS- UND REMISSIONSMESSUNGEN

Auftrag am Startfleck (µg)	*Transmissions-messung des Eluates*		*Remissionsmessung der Dünnschichtplatten*			
			Fläche $B_h \cdot H$		*Fläche planimetriert*	
	Verlust (%)	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*	*Verlust (%)*	*Fehler (± %)*
2.00	5.1	11.1	2.7	4.5	2.6	3.9
4.00	4.2	10.3	2.7	3.7	2.5	3.3
6.00	3.7	9.5	2.6	3.3	2.1	3.2
8.00	2.6	8.2	1.7	3.2	1.7	3.1
10.00	2.3	6.3	1.5	3.0	1.6	2.9

oder die relative Standardabweichung der Messungen, angegeben. Die Standardabweichung wurde nach

$$s = \pm \sqrt{\frac{\Sigma x_i^2 - N\bar{x}^2}{N-1}},$$

die relative Standardabweichung in % nach

$$s_{rel} = \pm \frac{s \cdot 100}{\bar{x}}$$

berechnet.

Den Tabellen ist zu entnehmen, dass einerseits die Substanzverluste bei der Remissionsmessung geringer sind als bei der Transmissionsmessung, und dass andererseits auch bei geringen Substanzkonzentrationen die Remissionsmessung noch zu genauen Ergebnissen mit einer relativen Standardabweichung unter 5% führt, während bei der Transmissionsmessung in diesem Fall mit einer relativen Standardabweichung, die *ca.* 10% beträgt, gerechnet werden muss. Wurden die von der Remissionsortskurve umschriebenen Flächen planimetriert, ist der Streubereich noch geringer. Er beträgt dann maximal 3.8% und erreicht damit einen Wert, wie ihn JORK[52] bei der quantitativen Auswertung von Trimethylxanthin und Acetylsalicylsäure durch direkte Absorptionsmessung und Berechnung nach der Kubelka–Munk-Funktion erhielt. Die

Messgenauigkeit war somit erheblich besser, als wie sie von STRUCK u.a.[3] bei der Bestimmung von Androgenen beschrieben wurde. Diese Autoren erreichten mit dem Chromatogramm-Spektralphotometer eine Messgenauigkeit von $s = \pm 10\%$.

Zur Untersuchung der Reproduzierbarkeit der Messungen auf verschiedenen DC-Platten wurden DC-Fertigplatten "Merck" im vorgesehenen Laufmittel zuerst blind entwickelt, dann in einer möglichst staub- und dampffreien Atmosphäre getrocknet. Auf die so vorbereiteten Dünnschichtplatten wurden die Glykosidgemische aufgetragen und entwickelt. Für jede Entwicklung wurde frisches Laufmittel in die Kammer gegeben. Nach der Entwicklung wurden die Platten mit dem Chromatogramm-Spektralphotometer ausgewertet und die Fläche unter der Remissionsortskurve, wie besprochen, planimetriert. Es wurden fünf Dünnschichtplatten auf diese Weise miteinander verglichen. Die Ergebnisse sind in Tabelle IV zusammengestellt.

TABELLE IV

REPRODUZIERBARKEIT (FEHLER IN %) DER MESSGENAUIGKEIT MIT DEM CHROMATOGRAMM-SPEKTRALPHOTOMETER AUF VERSCHIEDENEN DÜNNSCHICHTPLATTEN

Glykosid	*Aufgetragene Glykosidmenge (µg)*				
	2	*4*	*6*	*8*	*10*
Scillaren A ($R_F \times 100 = 16$)	5.3	4.9	4.1	3.7	3.6
Scillirosid ($R_F \times 100 = 22$)	5.4	5.2	4.6	4.1	3.9
Proscillaridin ($R_F \times 100 = 41$)	5.9	5.5	5.3	4.9	4.5

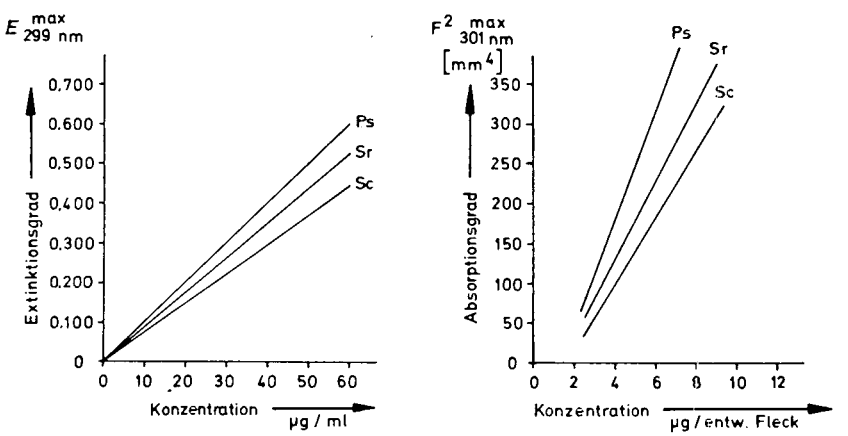


Fig. 4. Eichkurven für Transmissionsmessungen und Remissionsmessungen. Sc — Scillaren A; Sr — Scillirosid; Ps — Proscillaridin.

Die berechneten Fehlerbreiten liegen so günstig, dass es sinnvoll ist, Eichkurven aufzustellen, nach denen vor allem bei Routineuntersuchungen die Substanzkonzentrationen ermittelt werden können (Fig. 4).

Die Eichkurven wurden aus Punktmessungen durch Regressionsberechnungen[1] ermittelt. Für die Remissionsmessungen wurde auf der Ordinate die durch Planimetrieren ermittelte und quadrierte Fläche aufgetragen. Aus den Eichkurven ist zu ersehen, dass mit dem Chromatogramm-Spektralphotometer die Nachweisgrenze gegenüber der Transmissionsmessung des Eluats unveränderter Glykosidmoleküle um eine Zehnerpotenz erhöht ist. Die Eichkurven bei Remissionsmessung gehen nicht durch den Nullpunkt, und es ist daher zu erwarten, dass sie sich im Bereich sehr kleiner Konzentrationen nicht mehr linear verhalten. Das ist verständlich, weil die registrierten Remissionsmesskurven nicht exakt nach der Kubelka–Munk-Funktion, was die Ermittlung des absoluten Remissionsgrades vorausgesetzt hätte, ausgewertet wurden.

EXPERIMENTELLER TEIL

Für die zum Vergleich erforderlichen Transmissionsmessungen diente Kieselgel G "Merck" mit Fluoreszenzindikator als Adsorbens. Für die U.V.-Remissionsmessungen wurden DC-Fertigplatten Kieselgel "Merck" ohne Fluoreszenzindikator verwendet.

Selbstbeschichtete Platten wurden bei 140° aktiviert und bis zum Gebrauch im Exsikkator aufbewahrt, Fertigplatten im evakuierten Exsikkator über Phosphorpentoxid 24 Std. lang teilaktiviert.

Alle vorbereitenden Arbeiten wurden bei einer relativen Luftfeuchtigkeit unter 30% ausgeführt. Die Reinglykoside wurden in einer Mischung aus Methanol und Aethylacetat (50:50) gelöst und je nach Bedarf mit demselben Lösungsmittel für das Auftragen am Startfleck weiterverdünnt. Entwickelt wurde in allen Fällen mit dem Laufmittelgemisch Aethylmethylketon–Toluol–Wasser–Methanol–Eisessig (80:10:6:5:2). Die Laufstrecke betrug 15 cm.

Die Transmissionsmessungen wurden nach KRAUS u.a.[1] durchgeführt.

Für die Remissionsmessungen wurden die DC-Fertigplatten nach ihrer Entwicklung getrocknet und dann mit dem Chromatogramm-Spektralphotometer der Firma Zeiss ausgewertet. Die Auswertung erfolgte als Absorptionsmessung:

Anordnung	= M–Pr
Wellenlänge	= 301 nm
Spaltbild	= 14·0.5 mm
Verstärkung	= 10/1/IA
Geschwindigkeit	= Tisch 3
Schreiber	= 120 mm/min

Die von der Remissionsortskurve umschriebene Fläche wurde entweder durch Halbwertsbreite mal Höhe berechnet, bezüglich des Untergrundes korrigiert und dann quadriert oder planimetriert und die Planimetereinheiten ebenfalls quadriert.

DANK

Die Verfasser möchten an dieser Stelle Herrn Dr. W. TAUSCH in der Firma Carl Zeiss für wertvolle Beratungen und Auskünfte bestens danken.

ZUSAMMENFASSUNG

Es wurden verschiedene Methoden der direkten quantitativen Auswertung von Dünnschichtchromatogrammen für die Bestimmung von Bufadienoliden untersucht. Bei der Auswertung der Remissionsmessungen mit dem Chromatogramm-Spektralphotometer wurde für alle Bestimmungen ein Fehler von $s_{rel} < \pm 5\%$ gefunden. Die Methode schien daher nicht nur wegen des geringeren zeitlichen Aufwandes, sondern auch wegen der grösseren Genauigkeit den Transmissionsmessungen von Eluaten überlegen.

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CHROM. 3905

USE OF GEL FILTRATION IN THE ASSAY OF URINARY ENZYMES

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SUMMARY

Gel filtration of urine provides a simple and rapid, yet reliable, method for the separation of lactate dehydrogenase, alkaline and acid phosphatase, leucine aminopeptidase, arylsulphatase, and β-glucuronidase from interfering substances. It removes inhibitors more completely than dialysis and separates all spurious lactate dehydrogenase and alkaline phosphatase activities from the protein enzymes.

INTRODUCTION

The determination of urinary enzymes is increasingly being used for detection, differential diagnosis and following the course of urinary tract diseases[1–6]. However, various physiologically excreted ions and substances of low molecular weight interfere with the assay of enzymatic activity in "native" urine. Specifically, inorganic phosphate[7] and a urinary pigment[8] competitively inhibit phosphatases, saccharo-1,4-lactone[9] and various organic acids[10,11] inhibit β-glucuronidase, and two peptides of low molecular weight[12,13] inhibit lactate dehydrogenase. The ionic strength of the specimen may also influence enzyme activity[14,15]. Dialysis of urine against water previous to enzyme assay has been proposed to remove these interferences[1–5,16–20]. The present experiments investigate: (I) the efficiency of the commonly recommended method of dialysis in removing substances that interfere with enzyme analysis from urine; and (II) the advantages of a simple gel filtration technique as an alternative to dialysis. The six enzymes studied were selected because they have found wide clinical interest. Assay methods are partly modified micro versions of standard techniques.

METHODS

Samples

Urines were kept without additive at 4° until analysed (at the most 4 h), samples with abnormal protein or glucose content were discarded. Samples (10 ml) were

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centrifuged for 5 min at 4,500 r.p.m. Only the top 5 ml were used for analysis. If removed carefully this fraction is free of cells.

Dialysis

About 25 cm of dialysis tubing (7 mm diameter, 0.02 mm thickness. Type 8/32, Nr. 44104, Visking Corp., Chicago, Ill.) was filled with 5 ml of urine and tied safely at both ends. Dialysis was performed at room temperature against running tap water (12–15°) for various lengths of time, routine use was for 2 h (arylsulphatase was routinely dialyzed for 18 h at 4° against six changes of tap water)[17]. The water uptake during dialysis was determined by weighing and corrected for when calculating enzyme activities.

Gel filtration

Glass columns with a small dead space (inner diameter 1 cm, height 40 cm) were filled to a height of 14.5 cm with hydrated Sephadex G-50 (Pharmacia, Uppsala, Sweden) giving a total gel bed of 11.6 cm^3. A rubber sponge of 1 cm thickness was placed on top of the gel bed to facilitate sample application and to prevent the column from running dry[21]. Separation was performed at room temperature. Physiological saline (0.154 *M* NaCl) was used as eluant. To analyze the separation patterns, eluates were collected in 1 ml fractions. For routine analysis the following elution procedure was adopted: the sample (3 ml) was washed into the column with 1 ml of eluant followed by another 1 ml of eluant. The liquid emerging from the column up to that time (5 ml) was discarded. 6 ml of saline were now placed on the column, and the corresponding eluates collected for enzyme analysis. The column was then filled and rinsed with saline to prepare it for re-use.

Assay methods

Lactate dehydrogenase (EC 1.1.1.27). This was determined according to DORFMAN, AMADOR AND WACKER[18] with the following modification: 0.2 ml sample, 0.4 ml buffer-substrate solution (6.2 g sodium pyrophosphate, 2.34 ml 90% lactic acid, and 1.1 g NAD in 200 ml, pH 8.8) were used. Kinetic measurement was at 334 nm and 25°. One unit is defined as an increase of 0.001 O.D./min.

Alkaline phosphatase (EC 3.1.3.1). This was determined according to AMADOR, ZIMMERMAN AND WACKER[16] with the following modification: 0.2 ml sample and 0.4 ml buffer-substrate solution (2-amino-2-methyl-1-propanol-HCl, 0.75 *M*, pH 10.3; disodium-*p*-nitrophenylphosphate, 4.5 m*M*) were used. Kinetic measurement was at 405 nm and 25°. One unit is defined as an increase of 0.001 O.D./min.

Acid phosphatase (EC 3.1.3.2). 0.1 ml sample, 0.25 ml acetate buffer (0.2 *M*, pH 5.4), 0.25 ml substrate (disodium-*p*-nitrophenylphosphate, 12.6 m*M*) were used. After 10 min at 25° incubation was terminated by adding 2.5 ml NaOH (1 *N*), and the optical density measured at 405 nm. Results were corrected for reagent and sample blanks. Activity is reported in U/l.

Leucine aminopeptidase (EC 3.4.1.1). Here the L-leucine-*p*-nitranilide cleaving enzyme was determined according to NAGEL, WILLIG AND SCHMIDT[22] with the following modification: 0.02 ml sample, 0.6 ml Tris-buffer (0.05 *M*, pH 7.5), and 0.02 ml substrate (L-leucine-*p*-nitranilide, 0.025 *M*) were taken. Kinetic measurement was at 405 nm and 25°. Activity is reported in U/l.

Arylsulphatase (EC 3.1.6.1). Only arylsulphatase A was assayed. This was determined according to BAUM, DODGSON AND SPENCER[17] with the following modification: 0.2 ml sample, 0.1 ml buffer (1.0 *M* acetate buffer containing 0.5 m*M* $Na_4P_2O_7$ and 10% w/v sodium chloride, pH 5.0), and 0.1 ml substrate (2-hydroxy-5-nitrophenyl sulphate, 0.02 *M*) were used. After 60 min at 37°, incubation was terminated by adding 0.6 ml NaOH (1 *N*), and the optical density was measured at 515 nm. Results were corrected for reagent and sample blanks. One unit is defined as 1 μg/h/ml liberated 4-nitrocatechol.

β-Glucuronidase (EC 3.2.1.31). This was determined according to SZASZ[23]: 0.05 ml sample, 0.4 ml acetate buffer (0.2 *M*, pH 4.0) nad 0.05 ml substrate *p*-nitrophenyl-β-D-glucuronide, 50 m*M*) being used. After 300 min at 37°, incubation was terminated by adding 0.2 ml NaOH (0.5 *N*), and the optical density was measured at 405 nm. Results were corrected for reagent and sample blanks. Activity is reported in U/l.

Inorganic phosphate was determined by the vanadium–molybdate method[24]. Optical densities at 280 nm and at 405 nm were measured on a PMQ II spectrophotometer (Zeiss, Oberkochen, Württemberg, West Germany). All determinations were performed in duplicate.

RESULTS

Dialysis of urine

Fig. 1 shows the activities of leucine aminopeptidase, lactate dehydrogenase, acid and alkaline phosphatase, β-glucuronidase, and arylsulphatase before and after dialysis. In all instances the dialyzed samples had accelerated substrate turnover. Fig. 2 shows the effect of varying lengths of time of dialysis. There was an almost exponential removal of all inorganic phosphate but substances absorbing light at 280 nm and at 405 nm (urochromes) were only partly removed. The apparent activity

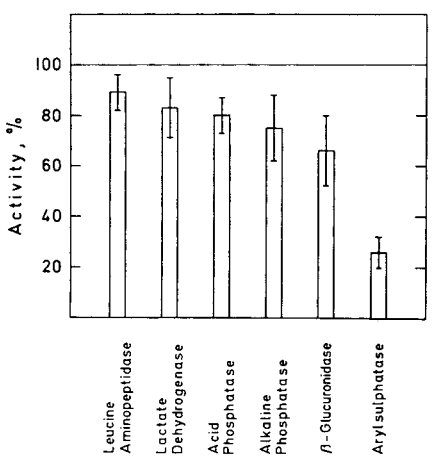

Fig. 1. Activities of six urinary enzymes, before and after dialysis. Activity before dialysis is expressed in per cent of the activity measured after dialysis. Ten urines were analyzed, mean values ± 1 standard deviation are plotted.

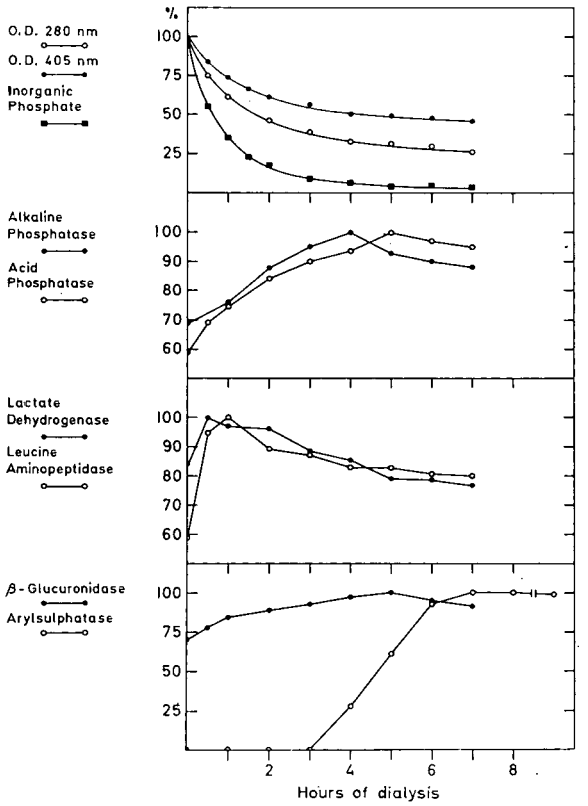

Fig. 2. Effect of varying the length of time of dialysis upon the optical densities at 280 nm and 405 nm, the concentration of inorganic phosphate, and the apparent activities of six enzymes in urine. Results are reported in percent of the highest measured value.

of the six assayed enzymes initially increased. Lactate dehydrogenase and leucine aminopeptidase reached peak activity during the first hour, alkaline phosphatase, acid phosphatase and β-glucuronidase from the fourth to the fifth hour, and arylsulphatase at the seventh hour. Subsequently the activities of all enzymes, except arylsulphatase, decreased.

Gel filtration of urine

The first 5 ml of the eluates were free of enzymes activity (see Fig. 3). The activity of all the assayed enzymes reached a peak in the next 6 ml with a maximum in the eighth ml. Lactate dehydrogenase and alkaline phosphatase showed a second peak of apparent activity with a maximum at the 16th and 17th ml, where substances absorbing light at 280 nm and inorganic phosphate also emerged. Heating to 95° abolished all lactate dehydrogenase and alkaline phosphatase activities found in the first peak, while the same treatment eliminated only a verying fraction of the apparent activities found in the second peak. Fig. 4 compares the separation of the two activity

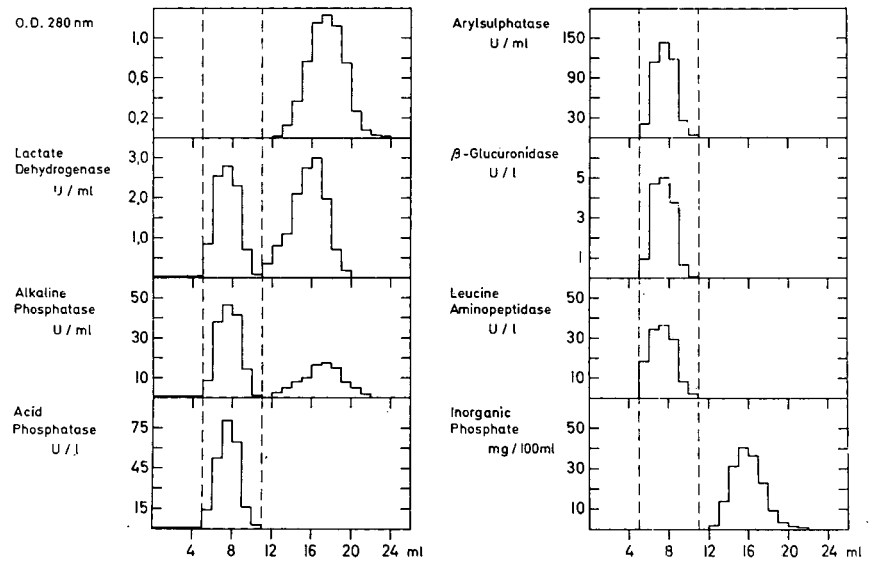

Fig. 3. Gel filtration of urine on Sephadex G-50, fine. Optical density at 280 nm (eluates diluted 1:20), apparent activities of six enzymes and concentration of inorganic phosphate in the eluates are shown. The dashed lines indicate the eluate fraction used in the routine procedure.

peaks on coarse and fine Sephadex G-50. In both instances the first peak emerged earlier on the coarse gel but only the fine gel separated the two peaks completely. This gel was used in all further experiments.

To test the accuracy of gel filtration urine samples had the interfering substances removed by a first gel filtration, then the samples were filtered a second time. 96% to 109% of the lactate dehydrogenase activity found after the first column passage

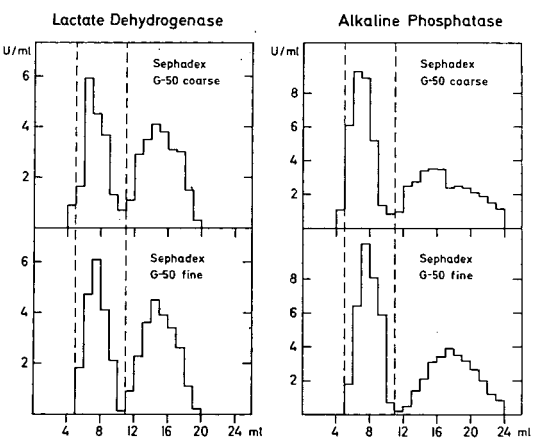

Fig. 4. Gel filtration of urine on Sephadex G-50, coarse (top), and Sephadex G-50, fine (bottom). The apparent activities of lactate dehydrogenase and alkaline phosphatase in the eluates are shown. The dashed lines indicate the eluate fraction used in the routine procedure.

TABLE I

ACCURACY OF GEL FILTRATION

Enzymatic lactate dehydrogenase activity was separated from interferences by gel filtration, subsequently the enzyme fraction was chromatographed a second time.

Urine	*Activity after first gel filtration U/ml*	*Activity after second gel filtration U/ml*	*Recovery %*
1	10.3	11.2	108.7
2	9.8	10.4	104.1
3	8.4	8.2	97.6
4	8.4	8.6	102.4
5	6.1	6.0	98.4
6	5.8	5.6	96.6
7	2.4	2.6	108.3
8	2.0	2.0	100.0
Mean	6.7	6.8	102.0

were thus recovered (Table I). Precision was tested by four replicate filtrations of the same urine on three different columns. The coefficient of variation of these twelve determinations (which includes the error inherent in the enzyme assay) was 2.4% (Table II).

Comparison of dialysis and gel filtration

Table III lists the enzyme activities of 25 urines after gel filtration and after dialysis. In all cases the activities of acid phosphatase, leucine aminopeptidase and β-glucuronidase were higher after gel filtration. The activity of arylsulphatase after 18 h dialysis and after gel filtration was not significantly different. (χ^2-test for positive and negative signs: $0.05 < P < 0.10$). The apparent activities of lactate dehydrogenase and alkaline phosphatase on the other hand were lower after gel filtration than after dialysis. For both activities substrate turnover, however, was linear after gel filtration, while substrate turnover after dialysis usually decreased with time. To investigate this difference a urine specimen was dialyzed for varying

TABLE II

PRECISION OF GEL FILTRATION

The arylsulphatase activity was assayed in twelve specimens of the same urine after gel filtration on three different columns. All results are reported in U/ml.

Column	*A U/ml*	*B U/ml*	*C U/ml*
Run 1	39.2	38.8	37.6
2	36.9	35.8	37.2
3	37.1	38.2	37.3
4	37.9	38.0	37.3
Mean for column	37.8	37.7	37.4
Grand mean		37.6	
Standard deviation		0.9	

TABLE III

COMPARISON OF THE APPARENT ACTIVITY OF SIX ENZYMES AFTER GEL FILTRATION (G) AND AFTER DIALYSIS (D)

The difference between the two results is expressed in per cent of the value found after gel filtration.

Urine	*Lactate dehydrogenase*			*Alkaline phosphatase*			*Acid phosphatase*		
	G U/ml	*D* U/ml	*Diff.* %	*G* U/ml	*D* U/ml	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %
1	2.8	5.4	+93	3.8	6.8	+79	22	13	−41
2	3.6	7.6	+111	16.5	20.0	+22	450	341	−24
3	1.1	4.8	+336	4.1	7.6	+85	227	209	−8
4	4.1	6.9	+68	9.2	8.5	−8	43	20	−53
5	11.1	9.9	−11	50.2	54.5	+9	301	272	−10
6	3.4	5.3	+56	9.0	7.0	−22	242	187	−23
7	4.9	5.1	+4	9.6	10.9	+14	37	34	−8
8	5.3	6.2	+17	18.0	22.1	+23	691	274	−60
9	4.0	5.6	+40	7.3	10.3	+41	237	174	−27
10	4.0	3.6	−10	4.9	7.3	+49	25	14	−44
11	5.3	6.1	+15	23.7	28.4	+20	629	319	−49
12	3.5	4.1	+17	6.7	7.9	+18	245	203	−17
13	3.5	3.8	+8	5.2	7.3	+40	30	24	−20
14	8.1	8.0	−1	29.3	34.7	+18	1018	850	−17
15	5.1	6.8	+33	9.7	12.5	+29	260	256	−2
16	2.6	4.6	+77	6.0	8.4	+40	20	16	−20
17	4.6	5.3	+15	11.7	16.5	+41	94	71	−24
18	21.2	21.6	+2	18.8	38.1	+103	204	187	−8
19	163.6	169.8	+4	16.9	13.6	−20	238	211	−11
20	2.9	4.5	+55	2.6	5.3	+104	23	18	−22
21	2.2	4.7	+114	15.3	25.7	+68	633	617	−3
22	4.4	6.2	+41	23.7	35.4	+49	63	50	−21
23	3.5	4.8	+37	6.9	9.4	+36	292	271	−7
24	1.5	4.2	+180	6.5	9.7	+49	29	22	−24
25	1.5	4.6	+207	6.5	9.0	+38	41	38	−7
Mean	11.1	12.8	+60	12.9	16.7	+37	244	188	−22

lengths of time before gel filtration (Fig. 5). As dialysis progressed the first peak retained its heat-labile lactate dehydrogenase and alkaline phosphatase activities unchanged while the apparent activities in the second peak decreased. After two hours dialysis some of the apparent activity in the second peak, however, still contributed to the total activity. Proceeding in reverse order, eluates containing the second peak were collected from gel filtration and dialyzed. Under these circumstances, dialysis removed all apparent lactate dehydrogenase and alkaline phosphatase activity within half an hour.

DISCUSSION

Dialysis increases the measurable urinary activity of all the six investigated enzymes by removing inhibitory substances. Different enzyme activities, however, reached their peak at different times during dialysis and subsequently decreased (except arylsulphatase); therefore, the same dialysis procedure cannot be applied to prepare a specimen for the assay of multiple enzymes. With gel filtration on Sephadex G-50,

Arylsulphatase			*β-Glucuronidase*			*Leucine amino-peptidase*		
G U/ml	*D* U/ml	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %
4.4	4.6	+4	0.20	0.12	−40	4.4	2.9	−34
29.0	28.2	−3	1.29	0.92	−29	9.9	5.6	−43
14.1	14.9	+6	0.43	0.26	−40	6.6	4.6	−30
9.4	5.0	−47	0.46	0.30	−35	4.6	4.0	−13
46.7	40.2	−14	2.08	1.21	−42	15.1	13.2	−13
16.4	6.6	−60	0.62	0.51	−18	6.0	4.7	−22
9.7	9.6	−1	0.46	0.29	−37	4.3	3.6	−16
19.8	17.9	−10	0.61	0.33	−46	7.9	7.0	−11
27.3	26.3	−4	0.55	0.45	−18	6.6	5.4	−18
3.3	3.7	+12	0.27	0.12	−56	4.1	2.8	−32
23.3	23.2	0	1.33	0.78	−41	7.9	7.0	−11
15.1	15.2	+1	0.54	0.43	−20	5.1	4.3	−16
15.9	16.6	+4	0.44	0.32	−27	7.1	4.1	−42
16.3	13.4	−18	1.12	0.62	−45	15.8	9.6	−39
7.6	7.5	−1	0.80	0.66	−17	10.1	6.6	−35
7.6	7.5	−1	0.56	0.45	−20	6.0	3.6	−40
10.3	7.0	−32	0.81	0.58	−28	7.5	5.5	−27
5.8	5.4	−7	2.42	1.66	−31	8.6	6.8	−21
8.9	8.7	−2	3.47	2.59	−25	10.1	7.6	−25
6.2	6.2	0	0.45	0.26	−42	6.0	2.3	−62
3.8	4.0	+5	1.31	0.79	−40	7.5	6.1	−19
1.6	1.2	−25	1.51	0.85	−44	6.4	4.8	−25
20.0	19.7	−1	1.54	1.05	−32	10.4	8.5	−18
5.1	5.2	+2	1.04	0.77	−26	6.2	3.6	−42
11.8	10.8	−8	0.66	0.48	−27	6.2	3.4	−45
13.6	12.3	−9	1.00	0.67	−33	7.6	5.5	−28

enzymes with a molecular weight over 30,000 emerge at the elution front and are also separated from smaller interfering substances. Even relatively large inhibitors and urochromes are thus completely removed from the urinary enzymes.

Although the eluant flow is more rapid on columns of coarse gel beads, the fine gel is preferable, since separation is sharper and elution from small columns remains sufficiently swift. Use of a sponge on top of the gel bed, or of a narrow constriction of the glass column immediately above the gel bed[25] prevents it from running dry. This makes constant column supervision unnecessary, and allows bulk elution for use in a clinical laboratory. Physiological saline is used as eluant, since proteins and other substances may adsorb to the gel when water is used. In the determination of multiple enzymes assay conditions differ, and buffers are, therefore, not suited as eluants. Sample volume and column dimensions are adjusted to minimize the dilution during column passage: 3 ml urine are eluated in 6 ml, a 1:2 dilution.

The method of gel filtration described is both accurate and precise. Higher urinary activities of acid phosphatase, β-glucuronidase and leucine aminopeptidase are found than after dialysis because gel filtration removes their inhibitors more

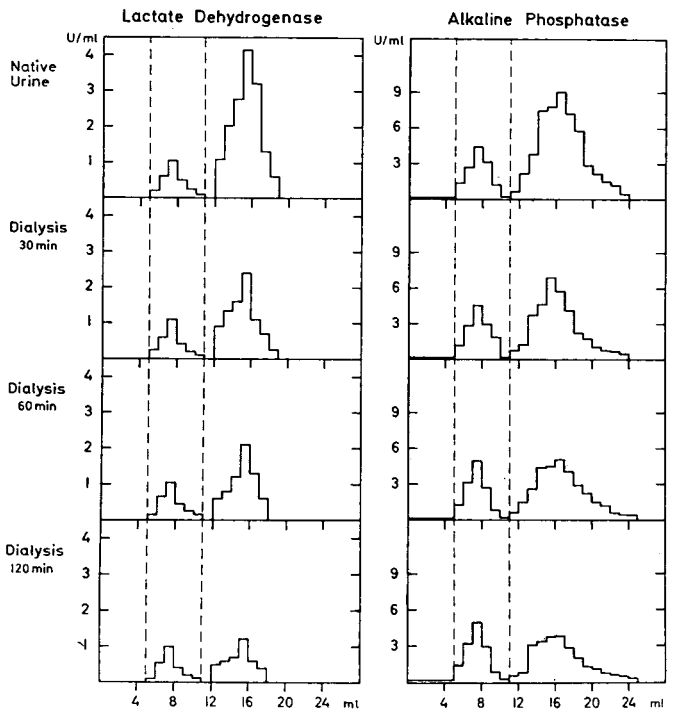

Fig. 5. Gel filtration of urine before and after dialysis of varying lengths of time. The apparent activities of lactic dehydrogenase and alkaline phosphatase in the eluates are shown.

completely. On the other hand the urinary activities of lactate dehydrogenase and alkaline phosphatase are higher after dialysis, as dialysis does not remove all the heat stable interfering substances causing spurious activity, which gel filtration separates from true enzyme activity. Since the sources and amounts of interfering substances in "native" urine are varied and unpredictable, changing from day to day even for the same individual[26], it is thus important that they be eliminated entirely.

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CHROM. 3918

FRAKTIONIERUNG VON CIGARETTENRAUCHKONDENSAT

II. GELFILTRATIONSCHROMATOGRAFIE VON FLUORESZIERENDEN INHALTSSTOFFEN EINER WASSERLÖSLICHEN FRAKTION AUS CIGARETTENRAUCHKONDENSAT

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(Eingegangen am 12. Dezember 1968)

SUMMARY

Fractionation of cigarette smoke condensate. II. Gel filtration chromatography of the fluorescing components of a water-soluble fraction of cigarette smoke condensate

The fluorescing components of the water-soluble fraction of cigarette smoke condensate were separated by chromatography on Sephadex gel. Thin-layer chromatographic investigations of the subfractions obtained demonstrated that the fluorescence of the water-soluble part of cigarette smoke condensate is due to the substances scopoletin, harman, norharman and at least 22 additional substances which occur in lesser quantities and which have not yet been identified. Scopoletin and the mixture of harman and norharman were separated from the other fluorescing substances by gel chromatography.

In the different subfractions, nicotine, nicotine-N-oxide, nicotinic acid as well as 17 other substances which react positively with BrCN/benzidine and which have not yet been completely identified, were found in the form of nonfluorescing components. These substances can also be partly separated from each other by gel chromatography. Therefore, chromatography on Sephadex gel appears to be a useful procedure for fractionating water-soluble components of tobacco smoke condensate.

EINLEITUNG

Bei der Fraktionierung von Cigarettenrauchkondensaten[1] fiel uns auf, dass in wasserlöslichen Fraktionen fluoreszierende Stoffe auftraten. Um diese Stoffe näher zu untersuchen, haben wir, unter Anlehnung an eine von DEMETRIOU *et al.*[2] beschriebene Methode, versucht, die wasserlöslichen Anteile des Cigarettenrauchkondensates an Sephadex G 15 Säulen aufzutrennen. Die Chromatografie an Sephadexgel scheint uns

eine brauchbare Methode zur Fraktionierung von wasserlöslichen Inhaltsstoffen des Rauchkondensates zu sein.

METHODEN

Vorbereitung und Betrieb der Säule

Das vernetzte Dextran Sephadex G 15 (Pharmacia), welches wir in den nachfolgend beschriebenen Versuchen benutzten, hatte eine Korngrösse von 40–120 μ und ein Wasseraufnahmevermögen von 1.5 ml/g. Für ein Säulenbettvolumen von 100 ml wurden *ca.* 33 g trockenes Gel benötigt.

Als Säule wurde ein Sephadexchromatografierohr Typ SR 25/45 mit einem Durchmesser von 2.5 cm und einer Länge von 45 cm verwendet. 33 g Sephadex G 15 wurden in 150 ml dest. Wasser aufgeschlämmt, über Nacht quellen gelassen und am nächsten Tag blasenfrei in die mit wenig dest. Wasser gefüllte Säule eingeschlämmt. Nach 5–10 min hatte sich das Gel abgesetzt. Das untere Säulenende wurde verschlossen und der obere Stempel der Säule vorsichtig mit offenem oberen Schlauchende soweit abgesenkt, bis die Unterkante des Stempels 1–2 mm von der Oberfläche des Gelbettes entfernt war. In dem Raum zwischen Gelbett und Stempel, sowie im oberen Schlauch waren keine Luftblasen mehr vorhanden. Das Ende des oberen Schlauches wurde in ein Niveaugefäss mit dest. Wasser gehängt und das Niveaugefäss so hoch gestellt, dass zwischen der Oberfläche des Gelbettes und dem Flüssigkeitsspiegel des Gefässes eine Höhendifferenz von 30 cm entstand. Die Säule wurde mit 100–250 ml dest. Wasser gewaschen und war dann betriebsfertig. Die Durchflussgeschwindigkeit betrug am Anfang 150–200 ml/Std. Nach einigen Stunden sank sie auf 100 ml/Std.

Etwa 30 mg der wasserlöslichen Rauchkondensatfraktion, gelöst in 1 ml Wasser, wurden auf die Säule aufgetragen.

Das Eluat wurde in Fraktionen von 10 ml mit einem Fraktionssammler Ultrorac 7000 der Firma LKB Stockholm aufgefangen.

Es wurden zwei verschiedene Elutionsmittel verwendet; zuerst 500 ml dest. Wasser und anschliessend 500 ml einer 5 m*M* Kochsalzlösung, die mit 1 *N* Salzsäure auf pH 4.0 eingestellt war. Das Gesamtelutionsvolumen betrug 1,000 ml. Nach Abschluss der Elution wurde die Säule mit 100 ml dest. Wasser gewaschen und war dann für einen neuen Versuch verwendungsfähig.

In der Praxis erwies es sich jedoch als günstiger, die Säule nach jedem Versuch neu einzuschlämmen, da die Laufgeschwindigkeit bei längerem Betrieb zu stark abnahm.

Automatische Registrierung der Fluoreszenz der Fraktionen

Die Fluoreszenz des Eluates wurde in einer Quarzdurchflusszelle von 1 cm Schichtdicke laufend mit einem Fluorimeter der Firma Zeiss verfolgt und auf einem Schreiber (Hitachi, Perkin Elmer Typ 159) registriert. Dadurch wurden Elutionsdiagramme erhalten, die die Fluoreszenz des Eluates in Abhängigkeit von dem Elutionsvolumen zeigten. Ein Beispiel für ein solches Elutionsdiagramm gibt die Fig. 1. Der Papiervorschub des Schreibers betrug 0.5 cm/min. Die Anregung der Fluoreszenz erfolgte bei einer Wellenlänge von 366 nm. Das emittierte Fluoreszenzlicht wurde über ein Sekundärfilter, welches ab 390 nm durchlässig war, auf einem Photomultiplier verstärkt und auf dem Schreiber registriert. Die Messanordnung wurde vor jedem Versuch mit einem Fluoreszenzglasstandard geeicht.

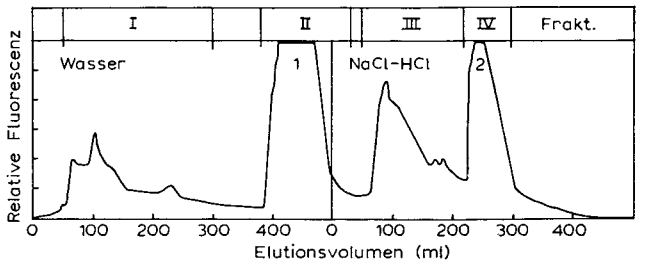

Fig. 1. Auftrennung einer wasserlöslichen Fraktion aus Cigarettenrauchkondensat an Sephadex G 15. Die Säule wurde zuerst mit 500 ml dest. Wasser und anschliessend mit 500 ml 0.005 *M* Natriumchlorid, pH 4.0 entwickelt. 1 = Scopoletin, 2 = Harman und Norharman.

Herstellung einer wasserlöslichen Fraktion aus Rauchkondensat

1 g Cigarettenrauchkondensat wurde zwischen 100 ml Äther und 100 ml Wasser verteilt. Die Ätherphase wurde noch dreimal mit je 100 ml Wasser extrahiert. Die Wasserphasen wurden vereinigt und auf dem Wasserbad bei 95° und schwachem Vakuum eingedampft. Die Ausbeute an wasserlöslichen Stoffen betrug 150 mg = 15%. 100 mg des Eindampfrückstandes wurden in 3 ml Wasser heiss gelöst und von einem geringen unlöslichen Anteil durch Filtrieren getrennt. Die so erhaltene Lösung wurde, wie oben beschrieben, auf die Säule aufgetragen.

Dünnschichtchromatografie

Für die Auftrennung und Identifizierung der Inhaltsstoffe in den Fraktionen der Gelchromatografie wurden folgende Systeme benutzt:

System A. Alkalische Kieselgelplatten. Laufmittel: Benzol–Äthanol (4:1). Die Platten wurden zweimal mit dem Laufmittel entwickelt. Zur Herstellung der alkalischen Platten wurden 20 g Kieselgel G nach Stahl in 50 ml 0.5 *N* KOH aufgeschlämmt, in einer Schichtdicke von 0.25 mm auf Glasplatten aufgetragen und an der Luft getrocknet.

System B. Neutrale Kieselgel-G-Platten. Schichtdicke: 0.25 mm. Laufmittel: Benzol–Äthanol (4:1). Die Platten wurden zweimal entwickelt.

System C. Neutrale Kieselgel-G-Platten. Schichtdicke: 0.25 mm. Laufmittel: Toluol–Essigsäureäthylester–Ameisensäure (5:4:1). Die Platten wurden zweimal entwickelt.

System D. Neutrale Kieselgelfertigplatten (Merck). Schichtdicke: 0.25 mm. Laufmittel: *n*-Butanol–Methanol–Benzol–Wasser (2:1:1:0.75).

System E. Neutrale Kieselgelfertigplatten (Merck). Schichtdicke: 0.25 mm. Laufmittel: Chloroform–Methanol–Ammoniak (60:10:1).

System F. Neutrale Kieselgelfertigplatten (Merck). Schichtdicke: 0.25 mm. Laufmittel: Chloroform–Methanol–Eisessig (60:10:1).

System G. Neutrale Kieselgelfertigplatten (Merck). Schichtdicke: 0.25 mm. Laufmittel: Benzol–Methanol–Ammoniak (82:18:0.8).

Sprühreagenzien für Dünnschichtchromatografie

Benzidin–Bromcyan. Nachweis von Nikotinalkaloiden und Pyridinderivaten.

1% Benzidin in Methanol. Anschliessend die Platte in Bromcyandampf entwickeln. Rotbraune, orange oder violette Flecken.

Gibbs Reagenz. Nachweis phenolischer Substanzen. 0.1% Dichlorchinonchlorimid in Methanol. Auf alkalischen Platten (DC-System A) zeigen sich nach dem Ansprühen, bei Vorhandensein phenolischer Substanzen, nach kurzer Zeit blaue Flecken.

Echtblausalz B. Nachweis von Phenolen und kupplungsfähigen Aminen. 0.5% 4,4'-Bis(2-methoxybenzoldiazoniumchlorid) in Methanol. Rotbraune bis violette Flecken.

Spektren

Die U.V.-Spektren wurden mit einem registrierenden Gerät 137 UV der Firma Perkin Elmer, die Fluoreszenzspektren mit einem registrierenden Fluorimeter ZFM4 der Firma Zeiss aufgenommen.

ERGEBNISSE

Unsere Untersuchungen ergaben, dass eine Auftrennung der wasserlöslichen Fraktion des Rauchkondensates an Sephadex G 15 möglich ist.

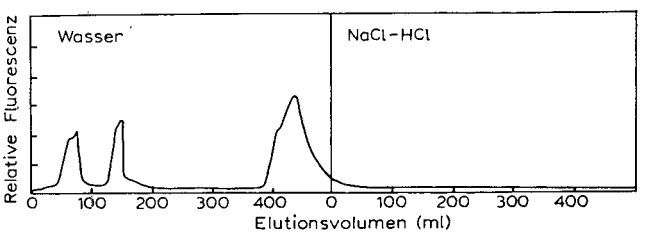

Fig. 2. Elutionsdiagramm eines wässrigen Tabakextraktes.

Fig. 1 zeigt ein typisches Elutionsdiagramm. Das Diagramm lässt erkennen, dass die Fluoreszenz der wasserlöslichen Fraktion auf mindestens vier Stoffe oder Stoffgruppen zurückzuführen ist. In Fig. 2 ist das unter den gleichen Bedingungen aufgenommene Elutionsdiagramm eines wässrigen Tabakextraktes dargestellt. Ein Vergleich mit Fig. 1 zeigt, dass wahrscheinlich ein Teil der fluoreszierenden Inhaltsstoffe der wasserlöslichen Fraktion des Rauches schon im Tabak selber vorkommt, ein anderer Teil jedoch erst während des Rauchprozesses gebildet wird.

Zur weiteren Charakterisierung der fluoreszierenden Stoffe wurden die im Fraktionssammler aufgefangenen Eluate zu vier verschiedenen Fraktionen vereinigt (Fig. 1) und dünnschichtchromatografisch untersucht. Aufgrund der verhältnismässig einheitlichen Form der Elutionsmaxima der Fraktionen II und IV vermuteten wir, dass in diesen Fraktionen nur jeweils ein fluoreszierender Stoff oder eine Stoffgruppe vorhanden sei.

Bei der stark fluoreszierenden Fraktion II bestätigte sich diese Annahme. Als fluoreszierender Stoff wurde *Scopoletin* nachgewiesen. Die Identifizierung erfolgte durch Vergleich der R_F-Werte in den Dünnschichtsystemen A, B und C und Vergleich der Fluoreszenz- und U.V.-Spektren mit denen von authentischem Scopoletin*.

* Scopoletin von der Firma Roth, Karlsruhe; nachträglich durch mehrfache DC gereinigt.

Die Fraktion IV enthielt als fluoreszierende Hauptbestandteile *Harman* und *Norharman*. Harman und Norharman konnten durch Dünnschichtchromatografie in dem System B getrennt werden. Die Fluoreszenzspektren und die R_F-Werte in den DC-Systemen A, B und C waren mit den Daten authentischer Harman- bzw. Norharmanproben* identisch.

Die Fraktion I enthielt nach Auftrennung im DC-System A, sieben fluoreszierende Substanzen. Davon lagen drei Substanzen in höherer Konzentration und die restlichen vier nur in Spuren vor.

Die Fraktion III wies, im DC-System A, zehn fluoreszierende Substanzen auf. Drei davon zeigten eine stärkere Fluoreszenz, die restlichen sieben fluoreszierten nur schwach.

Die Identifizierung dieser Substanzen gelang bisher noch nicht.

Erste Hinweise über weitere, nicht fluoreszierende Inhaltsstoffe in den Fraktionen I–IV erhielten wir durch Behandlung der Dünnschichtplatten mit verschiedenen Sprühreagenzien. Die Reaktion auf Pyridinderivate mit BrCN und Benzidin z.B. zeigte, dass die Bromcyan positiven Substanzen in den Fraktionen I und III angereichert waren. Die Fraktionen II und IV zeigten praktisch keine Reaktion mit BrCN/Benzidin. Die Fraktion I enthielt 13 Bromcyan-positive Stoffe. Drei davon konnten als *Nikotin*, *Nikotinsäure* und *Cotinin* identifiziert werden. Die Identifizierung erfolgte durch Vergleich der R_F-Werte mit denen von authentischen Substanzproben in den sieben DC-Systemen A, B, C, E, F, G und D. Die Konzentration des Nikotins in der Fraktion I war allerdings sehr gering.

Die Fraktion III enthielt neben sehr viel Nikotin geringe Mengen von sieben weiteren Bromcyan-positiven Stoffen. Neben dem *Nikotin* konnte noch ein Stoff als *Nikotin-N-Oxid* identifiziert werden. Die Identifizierung erfolgte durch Vergleich der R_F-Werte in den DC-Systemen A, B, C, E, F und G.

Die Reaktion mit Gibbs Reagenz und Echtblausalz B liess vermuten, dass ein Teil der Inhaltsstoffe der Fraktionen I und III phenolischen Charakter tragen. In beiden Fraktionen waren jeweils Substanzen zu sehen, die mit den beiden Reagentien eine positive Reaktion gaben (DC-System A und B). Die Fraktionen II und IV zeigten keine Reaktionen.

TABELLE I

IDENTIFIZIERUNG EINIGER INHALTSSTOFFE IN DEN FRAKTIONEN DER SEPHADEXSÄULE

Fraktion	*Inhaltsstoff*
I	Sieben fluoreszierende Stoffe. Nikotinsäure, Cotinin, Spuren Nikotin, zehn weitere Substanzen, die mit BrCN/Benzidin rot orange angefärbt werden. Phenolische Substanzen.
II	Scopoletin.
III	Zehn fluoreszierende Substanzen. Viel Nikotin, Spuren Nikotin-N-oxid. Sieben weitere Substanzen, die mit BrCN/Benzidin rot orange angefärbt werden. Phenolische Substanzen.
IV	Harman, Norharman. Fünf weitere fluoreszierende Stoffe in Spuren.

* Firma Fluka: Harman wurde durch mehrfache DC gereinigt.

Eine Übersicht über die bisher in den vier Fraktionen von uns nachgewiesenen Inhaltsstoffe gibt die Tabelle I. Die Untersuchung der noch nicht identifizierten Substanzen wird weiter von uns verfolgt.

Die Befunde, dass das Cumarinderivat Scopoletin von N-Heterocyclen wie Harman, die weniger basischen Pyridinderivate Nikotinsäure und Cotinin weitgehendst vom Nikotin und offensichtlich auch phenolische Stoffe in verschiedene Fraktionen getrennt werden können, bestätigen die von DEMETRIOU und Mitarbeitern[2] an Testmischungen gemachten Beobachtungen und weisen daraufhin, dass die Chromatografie an Sephadex-gel generall ein nützliches Verfahren zur Fraktionierung wasserlöslicher Anteile des Rauchkondensates und von Rauchkondensatfraktionen sein könnte.

ZUSAMMENFASSUNG

Die fluoreszierenden Inhaltsstoffe der wasserlöslichen Fraktion eines Cigarettenrauchkondensates konnten durch Chromatografie an Sephadexgel aufgetrennt werden. Die dünnschichtchromatografische Untersuchung der dadurch erhaltenen Unterfraktionen ergab, dass die Fluoreszenz des von uns untersuchten wasserlöslichen Anteiles des Cigarettenrauchkondensates auf die Substanzen Scopoletin, Harman, Norharman und mindestens 22 weitere in geringeren Mengen vorkommende noch nicht identifizierte Stoffe zurückzuführen ist. Scopoletin und das Gemisch aus Harman und Norharman wurden durch die Gelchromatografie in verschiedenen Fraktionen weitgehendst von den anderen fluoreszierenden Stoffen abgetrennt.

Als nichtfluoreszierende Inhaltsstoffe wurden in verschiedenen Fraktionen Nikotin, Nikotin-N-oxid, Cotinin, Nikotinsäure sowie 17 weitere mit BrCN/Benzidin-positiv reagierende, noch nicht näher definierte Substanzen gefunden. Auch diese Substanzen können teilweise durch die Gelchromatografie getrennt werden. Die Chromatografie an Sephadexgel scheint demnach ein brauchbares Verfahren für die Fraktionierung von wasserlöslichen Inhaltsstoffen des Tabakrauchkondensates zu sein.

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CHROM. 3915

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND ELUTION POSITION IN AN ANION EXCHANGE SYSTEM*

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SUMMARY

The elution positions of 114 reference compounds have been determined in the high-resolution anion exchange system being developed at the Oak Ridge National Laboratory for the analysis of body fluids. Compounds of the pyrimidine, amino acid, imidiazole, purine, pyridine, indole, quinaldic, and benzoic families of compounds and their derivatives elute in the approximate order of listing; however, extensive overlapping occurs. From consideration of the chemical family, the attached functional groups, the position of the attached functional groups, and the extent of conjugation, it is possible to make useful predictions of the elution positions of known compounds.

The regularities noted have been useful in predicting the elution position of known compounds and identifying compounds responsible for peaks. Predictions of peak positions are best made where related members of the same family have been tested.

INTRODUCTION

The identification of the compounds responsible for more than 150 peaks obtained by chromatography of human urine has been a significant problem in the application of the high-resolution, anion-exchange chromatograph in this laboratory[1,2]. Since the clinical evaluation of this analytical system at medical research establishments is imminent, the need for peak identification has become acute.

To date, specific compounds have been related definitely to 13 peaks, and tentatively to about 20 other peaks, found in urine chromatograms by the conventional techniques of co-chromatography plus mass spectrometry, nuclear magnetic resonance spectrometry, optical spectrometry, and chemical testing of separated fractions[3]. Characteristic of most of the identified compounds is the previous knowledge of their presence in large concentrations and their ultraviolet spectra. The identification of the remaining peaks will be difficult. The following factors contribute significantly to the difficulty: the presence of unknown compounds and conjugates, the large number of

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** Operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation.

chemical families represented, the incomplete resolution of chromatographic peaks, the small quantities of the separated compounds (in many cases less than 1 μg), the low ultraviolet absorptivities of some of the compounds, and the admixture of gradient salt (exceeding the concentrations of the unknown compounds in some instances by a factor of $> 10^6$, by weight).

Additional promising techniques that are now being applied to this identification problem include: the collection of large quantities of the separated compounds with larger anion exchange columns and other chromatographic media[4]; the use of more-volatile gradient salts to facilitate their removal from collected fractions; adaptation of the gas chromatograph as an adjunct to the mass spectrometer; hydrolysis of the urine conjugates prior to analysis; polarography; improved separation of the observed peaks through alteration of the ion-exchange resin, the gradient system and other operating parameters; and a study of the relationship between elution position and chemical structure of eluted compounds.

This paper, which discusses the relationship between elution position and chemical structure, is limited to descriptions of the structural differences of 114 compounds and how these differences are related to retention on the column. For these compounds, the published pK values offer only a rough guide to the order of elution. In their pioneer work in this field, VOLKIN AND COHN[5] reported similar experience; the factors that modify the order of elution have been discussed by SOBER AND PETERSON[6], and COHN[7]. A theoretical study of the system is hampered because of the large number of chemical families involved, the limited number of compounds tested in some families, and the interdependent operating variables that must be considered. These variables include the nonlinear concentration gradient of the eluent, the nonlinear liquid flow rate, the change in column temperature, and the observed variation of the pH of the eluent.

EXPERIMENTAL DATA

The elution positions of 114 compounds were determined by chromatographing solutions of reference compounds, using the high-pressure, anion-exchange chromatographic system previously described[1,2]. These compounds were selected because: (1) they were considered to be probable constituents of urine, (2) they give useful ultraviolet spectra, and (3) they are commercially available in a relatively pure form. The elution positions of a small number of compounds, principally amino acids, were determined with radioactive tracers.

The elution positions of the reference compounds, shown superimposed upon a chromatogram of a urine sample in Fig. 1, illustrate the general distribution of peaks and compounds. To facilitate the discussion of chemical structure, the chemical families are assembled in Tables I–XI in approximate order of elution and in the order of elution within families.

DISCUSSION

The compounds that exhibited little or no retention on the anion exchange column include all those listed in Table I (basic and neutral compounds); one member

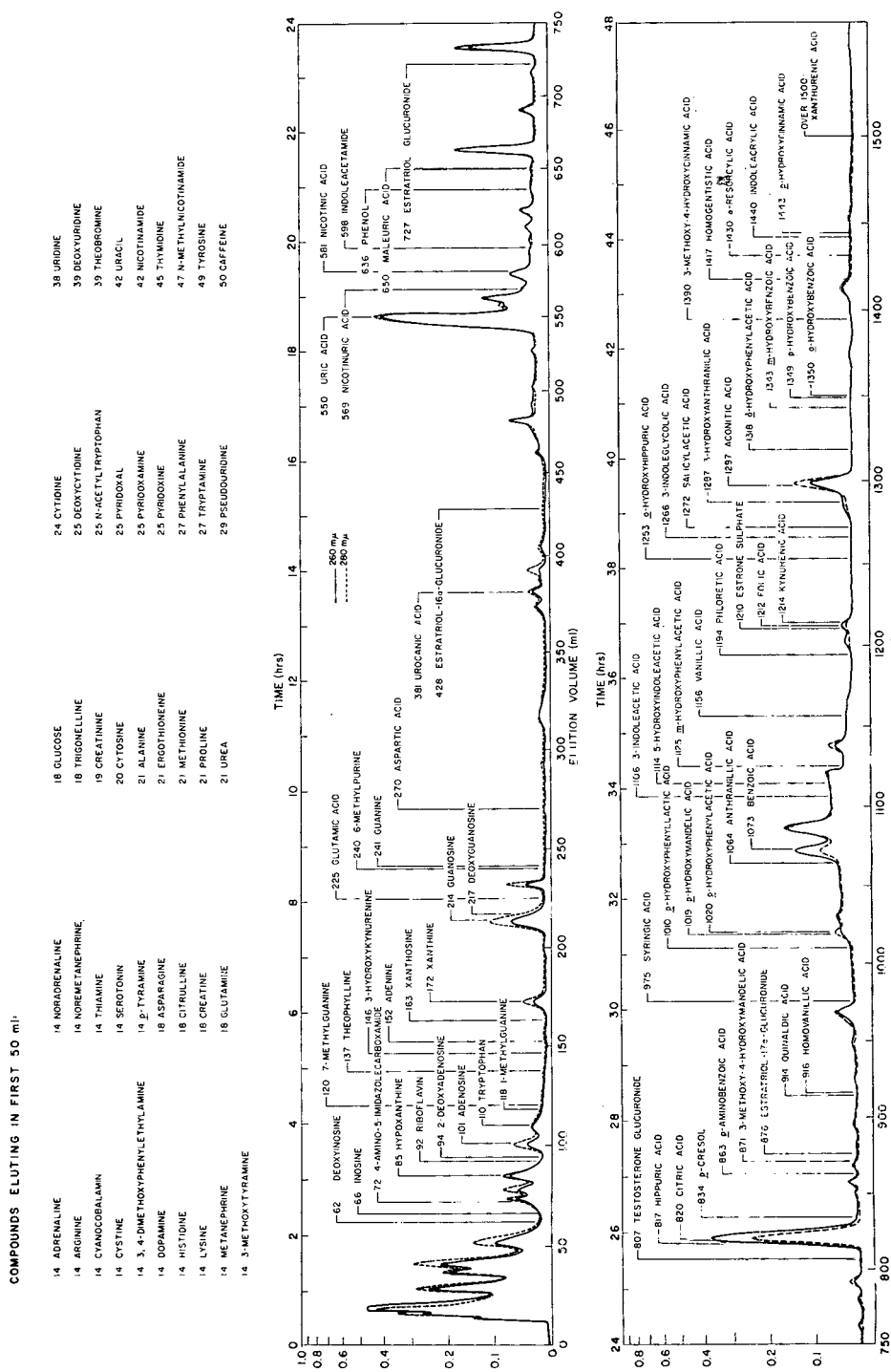


Fig. 1. A chromatogram of a urine sample showing position of known compounds, using urine reference sample No. 1.

each of the pyrimidine (Table II), imidazole (Table IV), and pyridine (Table VI) families; and a large number of the amino acids (Table III).

TABLE I

BASIC AND NEUTRAL COMPOUNDS

Compound	*Elution volume (ml)*
Adrenaline	14
Cyanocobalamin	14
3,4-Dimethylphenylethylamine	14
Dopamine	14
Insulin	14
3-Methoxytyramine	14
Metanephrine	14
Noradrenaline	14
Normetanephrine	14
p-Tyramine	14
Glucose	18
Urea	21

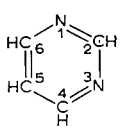
Basic and neutral compounds (Table I)

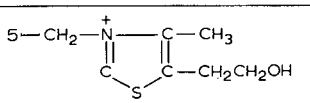
Aromatic bases and weakly ionized or neutral compounds were eluted at a single column volume, *i.e.* at 14 ml, and with no retention. In no case (except for the amino acids) did a compound having a carboxyl group or a carbonyl group with an α-hydrogen elute at 14 ml. However, many of the early-eluting compounds contained functional groups, such as hydroxyl, amide, imide, methoxy, etc., which apparently had no effect on their elution volumes. Urea (at 21 ml) and glucose (at 20 ml) were eluted later than might be expected. Thiamine (at 14 ml) and ergothioneine (at 21 ml), containing quaternary nitrogens, eluted near the breakthrough point, as expected.

TABLE II

PYRIMIDINE DERIVATIVES

Primary structure:



Compound	*Additions to pyrimidine structure*	*Elution volume (ml)*
Thiamine	2—CH_3, 4—NH_2, 	14
Cytosine	2=O, 4—NH_2	20
Cytidine	1—β-D-Ribofuranosyl, 2=O, 4—NH_2	24
Deoxycytidine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 4—NH_2	25
Pseudouridine	1—H, 2=O, 3—H, 4=O, 5—β-D-ribofuranosyl	29
Uridine	1—β-D-Ribofuranosyl, 2=O, 3=H, 4=O	38
Deoxyuridine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 3—H, 4—O	39
Uracil	1—H, 2=O, 3—H, 4=O	42
Thymidine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 3—H, 4=O, 5—CH_3	45

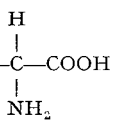
Pyrimidine derivatives (Table II)

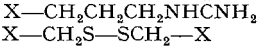
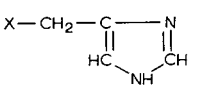
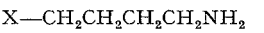
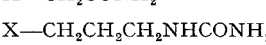
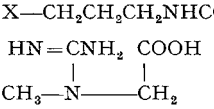
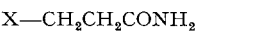
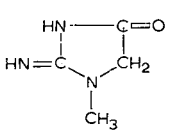
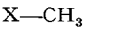
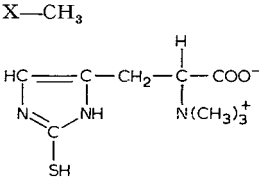
All members eluted early, with the relative position being strongly affected by the carbonyl group with an α-hydrogen. Three family members, each having one of these groups, eluted at 20–25 ml, whereas four other members, each having two of these groups, eluted at 29–45 ml. The inclusion of a carbohydrate structural element (and variations in that element) appears to shift the elution position by a few ml. As noted previously by COHN[8], the methyl group at position 5 in thymidine appears to favor later elution.

Riboflavin, while not a member of this family, resembles uridine and uracil structurally. Its large unsaturated cyclic structure appears to favor later elution (at 92 ml).

TABLE III

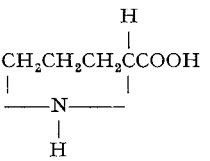
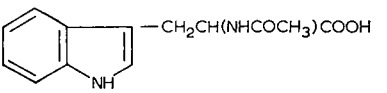
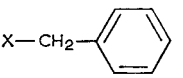
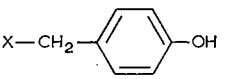
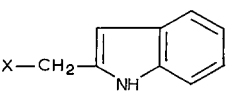
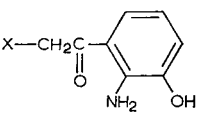
ORDER OF ELUTION OF AMINO ACIDS AND RELATED COMPOUNDS

Primary structure: X = 

Amino acid	*Structure*	*Elution volume (ml)*
Arginine		14
Cystine		14
Histidine		14
Lysine		14
Asparagine		18
Citrulline		18
Creatine		18
Glutamine		18
Creatinine		19
Alanine		21
Ergothioneine		21

(continued on p. 275)

TABLE III *(continued)*

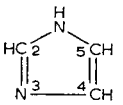
Amino acid	*Structure*	*Elution volume (ml)*
Methionine	X—$CH_2CH_2SCH_3$	21
Proline		21
N-Acetyltryptophan		25
Phenylalanine		27
Tyrosine		49
Tryptophan		110
3-Hydroxykynurenine		146
Glutamic acid	X—CH_2CH_2COOH	225
Aspartic acid	X—CH_2COOH	270

Amino acids (Table III)

The amino acids elute, as expected, in the following order: basic → aliphatic → aromatic → acidic. The aromaticity of phenylalanine (eluted at 27 ml), tyrosine (at 49 ml), and tryptophan (at 110 ml) favors longer retention times. The acetylation of the α-amino group of tryptophan reduces the elution volume from 110 to 25 ml. When the second carboxyl groups of glutamic acid (which is eluted at 225 ml) and aspartic acid (eluted at 270 ml) are blocked by amide groups to form glutamine and asparagine, respectively, the retention volume (18 ml) of each is approximately the retention volumes of the aliphatic monocarboxylic amino acids.

TABLE IV

IMIDAZOLE DERIVATIVES

Primary structure: 

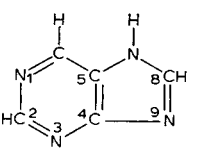
Compound	*Additions to imidazole structure*	*Elution volume (ml)*
Histidine	4-$CH_2CH(NH_2)COOH$	14
4-Amino-5-imidazole carboxamide	4-NH_2, 5-$CONH_2$	72
Urocanic acid	4-CH=CHCOOH	381

Imidazole derivatives (Table IV)

The imidazole structure appears to have less effect on retention on the column than other ring structures tested. Histidine elutes much earlier than the other aromatic amino acids, and urocanic acid elutes much earlier than comparable benzene derivatives (Table X) or the aliphatic unsaturated acids (Table VIII). The unsaturation in the side chain of urocanic acid, as in compounds of Table VIII and X, increases retention.

TABLE V

PURINE DERIVATIVES AND RELATED COMPOUNDS

Primary structure: 

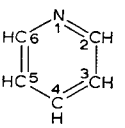
Compound	*Additions to purine structure*	*Elution volume (ml)*
Theobromine	1-H, 2 =O, 3-CH_3, 6 =O, 7-CH_3	39
Caffeine	1-CH_3, 2 =O, 3-CH_3, 6 =O, 7-CH_3	50
Deoxyinosine	1-H, 6 =O, 9-(2-deoxy-β-D-ribofuranosyl)	62
Inosine	6 =O, 9-β-*D*-ribofuranosyl	66
Hypoxanthine	3-H, 6 =O	75
Deoxyadenosine	6-NH_2, 9-(2-deoxy-β-D-ribofuranosyl)	94
Adenosine	6-NH_2, 9-β-D-ribofuranosyl	101
1-Methylguanine	1-CH_3, 2-NH_2, 3-H, 6 =O	118
7-Methylguanine	2-NH_2, 3-H, 6 = O, 7-CH_3	120
Theophylline	1-CH_3, 2 =O, 3-CH_3, 6 =O	137
Adenine	6-NH_2	152
Xanthosine	1-H, 2 =O, 3-H, 6 =O, 9-β-D-ribofuranosyl	163
Xanthine	1-H, 2 =O, 3-H, 6 =O	172
Guanosine	1-H, 2-NH_2, 6 =O, 9-β-D-ribofuranosyl	214
Deoxyguanosine	1-H, 2-NH_2, 6 =O, 9-(2-deoxy-β-D-ribofuranosyl)	217
6-Methylaminopurine	6-$NHCH_3$	240
Guanine	2-NH_2, 3-H, 6 =O	241
Uric acid	1-H, 2 =O, 3-H, 6 =O, 8 =O, 9-H	550

Purine derivatives (Table V)

As with the pyrimidine derivatives, the carbonyl group with an α-hydrogen strongly favors later elution. The most striking series is that of hypoxanthine (at 75 ml), xanthine (at 172 ml), and uric acid (at 550 ml), which have one, two, and three of these structural elements, respectively. When the α-hydrogen is replaced by α-methyl groups, as in theobromine, caffeine, and theophylline, these compounds elute earlier than the corresponding α-hydrogen analog (xanthine). An amine group in the 2 or 6 position favors later elution, as compared with the analogous carbonyl compounds; the methylamine groups appear more effective than the amine group (as in 6-methylaminopurine). As with the nucleosides of the pyrimidine family, these were separated from their base compounds only by a few ml.

TABLE VI

PYRIDINE DERIVATIVES

Primary structure: 

Compound	*Additions to pyridine structure*	*Elution volume (ml)*
Trigonelline	1-CH_3^+, 3-COO^-	15
Pyridoxamine	2-CH_3, 3-OH, 4-CH_2NH_2, 5-CH_2OH	25
Pyridoxal	2-CH_3, 3-OH, 4-CHO, 5-CH_2OH	25
Pyridoxine	2-CH_3, 3-OH, 4-CH_2OH, 5-CH_2OH	25
Nicotinamide	3-$CONH_2$	42
N-Methylnicotinamide	1-CH_3, 3-$CONH_2$	49
Nicotinuric acid	3-$CONHCH_2COOH$	569
Nicotinic acid	3-COOH	581

Pyridine derivatives (Table VI)

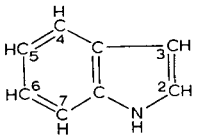
The basic and neutral members elute prior to 50 ml and the acid members at 550–600 ml. Trigonelline (which elutes at 15 ml) appears to be an exception; its carboxyl group may be neutralized by the quaternary nitrogen. The functional group in the third position increases retention in the order (of increasing effect): hydroxyl, carboxamide, and carboxyl. When the carboxyl group is conjugated, as in the case of glycine in nicotinuric acid, elution volume is decreased. The methyl group on the nitrogen atom improves the effect of the carboxamide in the third position, as in N-methylnicotinamide. From comparisons of the retentions of nicotinuric and nicotinic acids with those of hippuric and benzoic acids (Table X), it appears that the pyridine ring contributes to later elution less than the benzene ring does.

Indole derivatives (Table VII)

The elution volumes are, in most cases, approximately the same as those for analogous benzene derivatives (Table X). The acetamide, however, elutes later than might be expected.

TABLE VII

INDOLE DERIVATIVES

Primary structure: 

Compound	*Additions to indole structure*	*Elution volume (ml)*
Serotonin	3-$CH_2CH_2NH_2$, 5-OH	14
N-Acetyltryptophan	3-$CH_2CH(NHCOCH_3)COOH$	25
Tryptamine	3-$CH_2CH_2NH_2$	25
Tryptophan	3-$CH_2CH(NH_2)COOH$	110
3-Indoleacetamide	3-CH_2CONH_2	598
3-Indoleacetic acid	3-CH_2COOH	1106
5-Hydroxyindoleacetic acid	3-CH_2COOH, 5-OH	1114
3-Indoleglycolic acid	3-CHOHCOOH	1266
Indoleacrylic acid	3-CH=CHCOOH	1440

Aliphatic acids (Table VIII)

The polar carboxyl group and unsaturation cause the late elution.

TABLE VIII

ALIPHATIC ACIDS OTHER THAN AMINO ACIDS

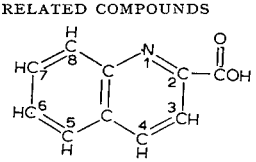
Compound	*Structure*	*Elution volume (ml)*
Maleuric acid	HOOC—CH=CH—CO—NH—$CONH_2$	650
Citric acid	HOOC—CH_2—COH(COOH)—CH_2—COOH	820
Aconitic acid	HOOC—CH=CH(COOH)CH_2—COOH	1297

Quinaldic acid derivatives (Table IX)

The elution volumes are approximately the same as those for analogous benzene derivatives (Table X). The hydroxyl groups at positions 4 and 8 favor later elution.

TABLE IX

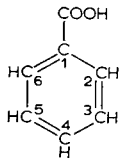
QUINALDIC ACID AND RELATED COMPOUNDS

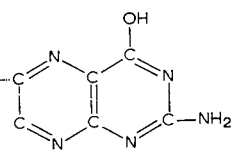
Primary structure: 

Compound	*Additions to quinaldic acid structure*	*Elution volume (ml)*
Quinaldic acid		914
Kynurenic acid	4-OH	1214
Xanthurenic acid	4-OH, 8-OH	>1500

TABLE X

BENZOIC ACID DERIVATIVES AND RELATED COMPOUNDS

Primary structure: 

Compound (acid)	*Substituted for —COOH*	*Additions to benzoic acid structure*	*Elution volume (ml)*
Phenol	—OH		636
Hippuric	—$CONHCH_2COOH$		817
p-Cresol	—OH	4—CH_3	834
p-Aminobenzoic		4—OH	863
3-Methoxy-4-hydroxy-mandelic	—CHOHCOOH	3—OCH_3, 4—OH	871
Homovanillic	—CH_2COOH	3—OCH_3, 4—OH	916
Syringic		3—OCH_3, 4—OH, 5—OCH_3	975
p-Hydroxyphenyllactic	—$CH_2CHOHCOOH$	4—OH	1010
p-Hydroxymandelic	—CHOHCOOH	4—OH	1019
p-Hydroxyphenylacetic	—CH_2COOH	4—OH	1020
Anthranilic		2—NH_2	1064
Benzoic			1073
m-Hydroxyphenylacetic	—CH_2COOH	3—OH	1125
Vanillic		3—OCH_3, 4—OH	1156
Phloretic	—CH_2CH_2COOH	4—OH	1194
Folic	—$CONHC(COOH)HCH_2HC(COOH)H$	4—$NHCH_2$— 	1212
o-Hydroxyhippuric	—$CONHCH_2COOH$	2—OH	1253
Salicylacetic		2—OCH_2COOH	1272
3-Hydroxyanthranilic		2—NH_2, 3—OH	1287
o-Hydroxyphenylacetic	—CH_2COOH	2—OH	1318
m-Hydroxybenzoic		3—OH	1343
p-Hydroxybenzoic		4—OH	1349
o-Hydroxybenzoic		2—OH	1350
3-Methoxy-4-hydroxy-cinnamic	—C(H)=CHCOOH	3—OCH_3, 4—OH	1390
Homogentisic	—CH_2COOH	2—OH, 5—OH	1417
α-Resorcylic		3—OH, 5—OH	1430
p-Hydroxycinnamic	—C(H)=CHCOOH	4—OH	1443

Benzene derivatives and related acidic compounds (Table X)

Basic and neutral derivatives that elute without retention on the column are discussed above (Table I). In an anion-exchange system, the thermodynamic stability of the anionic species (acid strength of the aromatic acids) (Table X) studied should be related to chemical structure and to retention on the anion-exchange column.

Since functional groups influence the reactivity or equilibria through inductive (electrostatic), resonance (conjugation), and steric effects[8], it should be possible to correlate elution volumes of aromatic acids with the functional groups present on the molecules. With some exceptions, such correlations appear in this family of compounds.

Benzoic acid, eluting at 1073 ml, may be considered as the base compound for the discussion that follows. The phenyl ring, through conjugation and induction, contributes to the stability of the benzoate ion; for example, benzoic acid was observed to elute later than the aliphatic acids (with the exception of aconitic acid). Compounds with functional groups that decrease the stability of the benzoate ion would be expected to elute earlier than benzoic acid; conversely, the compounds with functional groups that increase the stability should elute later. The compound *p*-aminobenzoic acid (eluted at 863 ml) illustrates the reduction of stability through resonance of the amino group in the para position, and the compound *o*-aminobenzoic acid (anthranilic acid), which is eluted at 1064 ml, shows increased stability through hydrogen bonding of the amino group in the ortho position. The inductive power of a meta hydroxyl group for stabilizing the anion is evident in the later elution of 3-hydroxyanthranilic acid (at 1278 ml).

On the basis of acid strength, the monohydroxy isomers of benzoic acid should elute in the sequence *p*-hydroxybenzoic acid, benzoic acid, *m*-hydroxybenzoic acid, and *o*-hydroxybenzoic acid. However, the sequence was found to be: benzoic acid (at 1073 ml), *m*-hydroxybenzoic acid (at 1343 ml), *p*-hydroxybenzoic acid (at 1349 ml), and *o*-hydroxybenzoic acid (at 1350 ml). The mono-hydroxy isomers of phenylacetic acid did elute in the predicted sequence of *p*-hydroxy (at 1020 ml), *m*-hydroxy (at 1125 ml), *o*-hydroxy (at 1318 ml). The high ionic strength of the eluting buffer (4–6 *M*) may affect the relative stabilities of the hydroxybenzoic acids more strongly than those of the hydroxyphenylacetic acids. The inductive effect of a *m*-hydroxy group on acid strength can be further illustrated with α-resorcylic acid (3,5-dihydroxybenzoic acid), which elutes at 1430 ml, as compared with *m*-hydroxybenzoic acid, which elutes at 1343 ml.

It has been shown above that the amine group in the ortho position of anthranilic acid, is capable of hydrogen bonding with the carboxyl group, thereby causing later elution. This effect was also noted with the hydroxyl groups of *o*-hydroxybenzoic acid and homogentistic acids. The hydroxyl group is more effective than the amino group in this respect; *i.e.*, the ortho hydroxyl compounds elute later than the ortho amino compounds.

The introduction of a methylene unit between the phenyl ring and the carboxyl group disrupts conjugation and results in acid weakening; this can be illustrated by the earlier elution of *p*-hydroxyphenylacetic (at 1020 ml) as compared with *p*-hydroxybenzoic acid (1349 ml) and by the earlier elution of homovanillic acid (at 916 ml) as compared with vanillic acid (at 1156 ml). An ethylene unit inserted between the phenyl and the carboxyl groups increases conjugation and results in acid strengthening, as illustrated by the late elution of 3-methoxy-4-hydroxycinnamic acid (at 1390 ml) and *p*-hydroxycinnamic acid (at 1443 ml), as compared with 3-methoxy-4-hydroxybenzoic (vanillic) acid (at 1156 ml) and *p*-hydroxybenzoic (1349 ml) acids.

Phenol and *p*-cresol, which are considered to be weak acids, are included as benzoic acid derivatives. As expected, they elute later than all basic and neutral

compounds but before most aromatic acids. *p*-Cresol, normally considered a weaker acid than phenol, unexpectedly elutes later than phenol.

TABLE XI

STEROIDS

Compound	*Elution volume (ml)*
Estratriol-16-α-glucuronide	428
Estratriol-3-glucuronide	727
Testosterone glucuronide	807
Estratriol-17-α-glucuronide	876
Estrone sulfate	1210

Steroid conjugates (Table XI)

The carboxyl group of the glucuronic acid moiety and the heterocyclic structure of the steroid moiety appear to be related to the later elutions of this family. The relatively late elution of the estrone sulfate probably results from the presence of the acid sulfate group.

CONCLUSION

The study of elution position as a function of chemical structure, rather than of pK values, was initiated for several reasons. First, the pK values are not available for many of the biological compounds of interest and, where available, may not be measured at the condition of interest. Second, as demonstrated by VOLKIN AND COHN[5] and SOBER AND PETERSON[6], the order of elution frequently does not follow the pK values, even in the case of simple systems. Third, the structure study complements other structural identification methods such as mass spectrometry and N.M.R. Finally, the method allows a small segment of the chromatogram to be considered so that linear correlation among family members becomes feasible. For example, the elution position of caffeine (at 50 ml) was predicted from the knowledge of related compounds in Table V.

A number of relationships that are consistent with accepted ideas of mechanism and structure[9] have been noted. In general, basic compounds elute first, followed by the neutral and then the acid compounds. Functional groups affect elution position most strongly in the case of acid compounds. Functional groups do not affect most basic compounds, which elute without retention; however, they do contribute to the separation within families of pyrimidines and purines and their nucleosides. Nucleosides differ only slightly from their bases in retention. It has been postulated that the retention of these compounds results from the affinity of the polystyrene matrix of the resin for these compounds[7]. COHN[7] considered purines to be more "benzenoid" than the pyrimidines and reported that alkylation increases affinity for the resin. The separations reported may be due to nonpolar binding, and the functional groups, depending on how they affect the nonpolar binding, may affect the elution position.

The amino acids elute earlier than most acid compounds. The basic and neutral

members elute with very little retention. Aromaticity, additional carboxyl groups, and additional hydroxyl groups increase the acid nature of the compounds and increase the retention volumes.

Indole and quinaldic derivatives and benzene rings make equivalent contributions to retention; imidazole and pyridine rings make less contribution. The basic and neutral aromatic compounds elute with little retention. The acid compounds are significantly retained; functional groups increase or decrease retention, depending on the nature of the group and its structural position on the molecule.

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Short communication

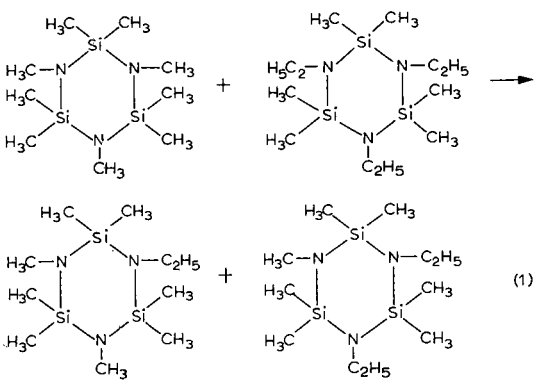
CHROM. 3931

Redistribution reactions of N-alkylcyclotrisilazanes

In recent years there has been a growing interest in silicone heterocycles in which silicon atoms alternate with heteroatoms. Redistribution reactions of this type of compound involving ring–chain equilibria have been studied in some detail[1]. However, apart from ring expansions and contractions[1,2] (usually acid or base catalyzed), little work has been presented relating to ring–ring equilibria. The redistribution of nonamethylcyclotrisilazane with hexamethylcyclotrisilthiazanes to form two new cyclotrisilthiazanes was reported recently[3], and full quantitative data were obtained for this reaction[4].

This communication presents evidence for the interchange of N-alkyl groups between cyclotrisilazanes to form new mixed N-alkylcyclotrisilazanes. This type of reaction is analogous to the exchange of labile B-alkyl groups between borazoles observed by NEWSON *et al.*[5].

Redistribution of N-alkyl groups was found to occur when equimolar quantities of tris-(N-methyl)-hexamethylcyclotrisilazane ($N^1N^1N^1$) and tris-(N-ethyl)-hexamethylcyclotrisilazane ($N^2N^2N^2$) were heated together in chloroform in sealed tubes for several hours at 140° (Eqn. 1).



(1)

Analysis of the reaction products was carried out using a Pye F. 104 gas chromatograph, with a flame ionization detector, and 6 ft. × $\frac{1}{4}$ in. O.D. column containing as a stationary phase 12% w/w Silicone Gum SE-30 on treated Celite. The column temperature was 180°. The chromatogram of the redistributed mixture showed the formation of two new compounds with retention volumes intermediate between those of ($N^1N^1N^1$) and ($N^2N^2N^2$). These new compounds were not formed when either ($N^1N^1N^1$) or ($N^2N^2N^2$) were heated alone (Fig. 1). A plot of log retention time *vs.* carbon number for the four compounds resulted in a straight line, as expected for an homologous series of this nature.

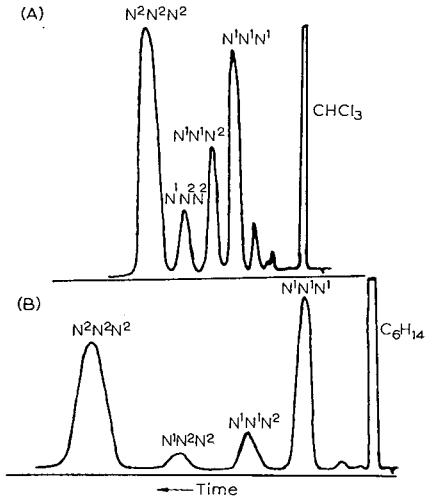
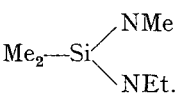


Fig. 1. Chromatograms obtained from the $N^1N^1N^1$–$N^2N^2N^2$ redistribution in chloroform (A) and hexane (B).

The GLC data were supported by the proton resonance spectrum of the redistributed mixture. The original mixture of ($N^1N^1N^1$) and ($N^2N^2N^2$) gave rise to five resonances, with values corresponding to those given in the literature[5]. After equilibration, new resonances were observed, due to silyl methyl protons in the environment



This type of reaction has also been found to occur in systems involving tris-(N-propyl)-hexamethylcyclotrisilazane ($N^3N^3N^3$). Preparation of cyclotrisilazanes with large N-alkyl groups is difficult or in some cases impossible[6,7], and it was not possible to obtain ($N^3N^3N^3$) in an extremely pure condition. However, mixtures containing this compound underwent redistributions with both ($N^1N^1N^1$) and ($N^2N^2N^2$) giving predicted gas chromatogram patterns, and linear plots of log retention volumes *vs.* carbon number.

A detailed account of these redistribution reactions will be presented later.

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Notes

CHROM. 3949

Gas chromatography and mass spectroscopy of plant phenolics and related compounds*

Gas–liquid chromatography (GLC) of trimethylsilyl ethers of sugars and other polyhydroxy compounds[1–3], phenolic acids[4–7], anthroquinones[8], phenols[9,10] and their glycosides have been reported by many workers. HORII *et al.*[11,12] have successfully separated some aromatic acids and Krebs-cycle acids as trimethylsilyl (TMS) derivatives. In addition, the results of BOLAN AND STEELE[13], STALLING *et al.*[14] suggest that GLC has a wide application for analysis of mixtures encountered in biological extracts.

When GLC is used in conjunction with mass spectroscopy the molecular weight of unknown TMS derivatives of a mixture can be determined. The application of these techniques is receiving considerable attention and this communication presents the results obtained with mixtures of TMS-ethers of 28 phenolic compounds and 4 phenolic glycosides.

Experimental

Materials. All compounds used were commercially available. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Applied Science Co. Reagent grade pyridine (Baker) which had been dried over 4A molecular sieve and distilled under N_2 atmosphere was used.

Trimethylsilylation. Five–six mg of a component was weighed for each mixture prepared and placed in a sealed vial under N_2 atmosphere. One ml of freshly prepared reagent containing HMDS, TMCS and pyridine (3:1:9) was injected into each vial. The reaction mixture was shaken vigorously for 30 sec and then allowed to stand at room temperature for at least 5 min. An aliquot from each mixture was injected directly into the gas–liquid chromatograph.

Gas–liquid chromatography. Analyses were performed on a Hewlett-Packard instrument (F & M, model 810-DR-12) equiped with dual glass columns, flame ionization and thermal conductivity detectors (only flame ionization was used). Glass columns with 1:1 effluent splitters were 4.0 ft. long (4 mm I.D.) and packed with Chromosorb Q (60–80 mesh) coated with 3% OV-1 (Applied Science Co.).

The carrier gas (He), H_2, and air flow rates were 50, 42, and 485 ml/min, respectively. The injection port and flame ionization detector temperatures were both 310°. These conditions were constant during the entire analyses. The column temperature was programmed as indicated in each figure.

Gas chromatography and mass spectroscopy. An LKB 9000 gas chromatograph–mass spectrometer (LKB-Produkter AB Stockholm-Brommal, Sweden) was used to determine the mass of the parent ion of each TMS ether derivative.

The TMS ether derivatives were chromatographed as above except thermal conductivity and 8.0 ft. columns were employed. The effluent of each component in a

* Journal paper No. 3543 of the Purdue Agricultural Experimental Station.

mixture entered directly into the mass spectrometer and mass spectra were obtained for each compound.

The mass spectrometer was operated at an electron energy of 20 eV, accelerator voltage of 3.5 kV, and an ion source temperature of 280°. A scan speed of 100 *m/e*/sec was used. The molecular separators were maintained at 275°.

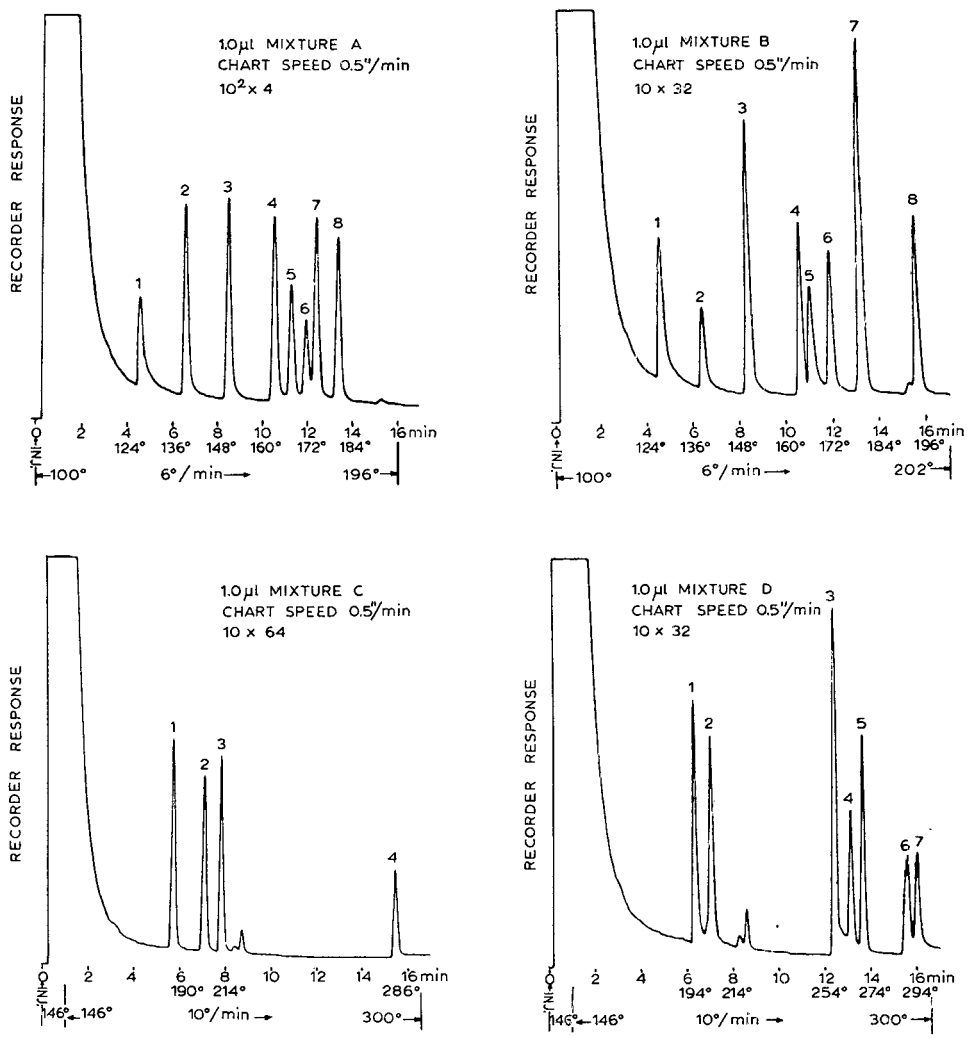

Fig. 1. Gas chromatographic separation of the TMS ether derivatives. The amount injected, chart speed, range and attenuation are described in the legends. Mixture A: 1 = coumarin, 2 = cinnamic acid, 3 = *p*-hydroxyphenylacetic acid, 4 = *p*-hydroxyphenylpropionic acid, 5 = *o*-coumaric acid, 6 = 3,4-dihydroxyphenylacetic acid, 7 = *m*-coumaric acid, 8 = *p*-coumaric acid. Mixture B: 1 = coumarin, 2 = vanillin, 3 = *p*-hydroxybenzoic acid, 4 = vanillic acid, 5 = umbelliferone, 6 = 3,4-dihydroxyphenylacetic acid, 7 = quinic acid, 8 = ferulic acid. Mixture C: 1 = quinic acid, 2 = *p*-hydroxyphenylpyruvic acid, 3 = caffeic acid, 4 = chlorogenic acid. Mixture D: 1 = scopoletin, 2 = esculetin, 3 = phloretin, 4 = naringenin, 5 = catechin, 6 = quercitin, 7 = myricetin. Mixture E: 1 = arbutin, 2 = esculin, 3 = phloridzin. Mixture F: 1 = chalcone, 2 = 2-hydroxychalcone, 3 = phloretin, 4 = phloridzin.

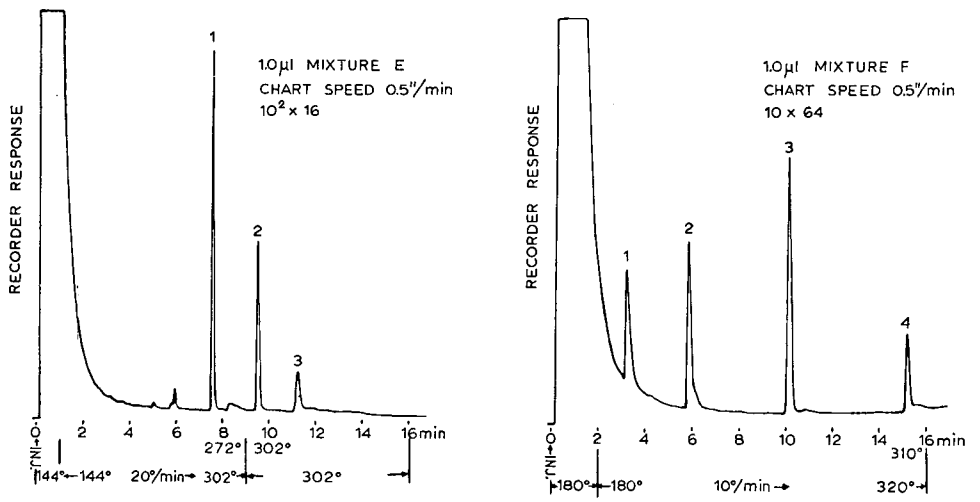

Fig. 1 *(continued)*.

TABLE I

PARENT ION OF THE TMS ETHER DERIVATIVES

TMS ether derivative	*Parent ion (m/e)*	*TMS ether derivative*	*Parent ion (m/e)*
Mixture A		*Mixture D*	
Coumarin	146	Scopoletin	264
Cinnamic acid	220	Esculetin	322
p-Hydroxyphenylacetic acid	296	Phloretin	562
p-Hydroxyphenylpropionic acid	310	Naringenin	488
o-Coumaric acid	308	Catechin	650
3,4-Dihydroxyphenylacetic acid	384	Quercitin	662
m-Coumaric acid	308	Myricetin	750
p-Coumaric acid	308		
		Mixture E	
Mixture B		Arbutin	632
Coumarin	146	Esculin	700
Vanillin	224	Phloridzin	940
p-Hydroxybenzoic acid	282		
Vanillic acid	312	*Mixture F*	
Umbelliferone	234	Chalcone	208
3,4-Dihydroxyphenylacetic acid	384	2-Hydroxychalcone	296
Quinic acid	552	Phloretin	562
Ferulic acid	338	Phloridzin	940
Mixture C		*Miscellaneous*	
Quinic acid	552	Pomiferin	638
p-Hydroxyphenylpyruvic acid	324	Solanidine	469
Caffeic acid	396		
Chlorogenic acid	786*		

* Did not give parent ion.

Results and discussion

The results of the gas chromatographic separation of the TMS ether derivatives are shown in mixtures A–F (Fig. 1). In mixtures A and B coumarin served as an internal standard since it does not have a hydroxyl group. All trimethylsilylated hydroxy-compounds were resolved from each other as sharp peaks. The retention time increased with the number of hydroxyl substituents or the molecular weight. The resolution of mixture A demonstrates that it is possible to separate closely related compounds and to resolve the isomers of coumaric acid.

Table I depicts the parent ion of the TMS ether derivatives. The observed *m/e* value agrees with the calculated molecular weight for each component. The TMS ether derivatives are thermally stable under the experimental conditions and each peak represents a completely trimethylsilylated compound.

The simultaneous use of gas chromatography and mass spectroscopy provides a powerful tool for the separation and identification of components in extracts of biological systems[1–3]. Mixture F contained known intermediates in the biosynthesis of the phenolic glycoside, phloridzin.

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CHROM. 3937

Identification of organophosphate pesticides by gas chromatography with the flame photometric detector*

Determination of retention times on gas chromatographic columns of different polarity is one technique for compound identification[1,2]. The combination of this technique with the use of a specific detector increases the reliability of the identification. Detectors which have been used for detection of organophosphate pesticides include the electron affinity detector[3], the microcoulometric detector[2], the thermionic detector[2], and the flame photometric detector[4]. The flame photometric detector provides the best combination of sensitivity and selectivity. Since the flame photometric detector will give a response for compounds containing both phosphorus and sulfur, it is especially well suited for the detection and identification of pesticides which contain these two elements.

In a recent study of pesticides in air, details of which will be published elsewhere, gas chromatography was selected for the analysis of samples primarily because the levels in most cases were too low to permit detection and identification by other methods. Both chlorinated pesticides and organophosphate pesticides were specifically sought; the latter included methyl parathion, malathion, and parathion. In addition, if any unknown organophosphate pesticides were detected, they had to be identified.

The approach selected for the detection and identification of organophosphate pesticides in this study consisted of measurement of retention times on two columns of different polarity plus detection of the compounds with the flame photometric detector in both the phosphorus and sulfur modes. This approach was especially useful for identifying organophosphates in three different types of samples. A description of the approach and of the applications follows.

Gas chromatographic conditions

A Micro-Tek GC 2500R gas chromatograph containing a Micro-Tek flame photometric detector equipped for use in either the phosphorus or the sulfur mode

TABLE I

RELATIVE RETENTION TIMES FOR ORGANOPHOSPHATE COMPOUNDS

Temperature, 140°; nitrogen carrier gas flow rate 100 ml/min. Methyl parathion retention times: 12 min on QF-1, 10 min on OV-1.

Compound	*Relative retention time*	
	QF-1	*OV-1*
Phorate	0.17	0.42
Tri-*n*-butyl phosphate	0.27	0.39
Methyl parathion	1.00	1.00
Malathion	1.10	1.58
Parathion	1.42	1.58
S,S,S-Tributyl phosphorotrithioate	1.15	3.18

* Paper presented before the Division of Agricultural and Food Chemistry, 157th Meeting, American Chemical Society, Minneapolis, Minn., April, 1969.

was employed. Glass columns 1 m long by 6 mm O.D. were used; one column was packed with 5% QF-1 (Chemical Research Services, Inc.) on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.) and the other column was packed with 5% OV-1 (Supelco, Inc.) on 100–120 mesh Gas-Chrom Q. Relative retention times for several organophosphate compounds on these columns are given in Table I. The column oven was set at temperatures from 140° to 170°; the higher temperatures were used in confirming runs for S,S,S-tributyl phosphorotrithioate (DEF, Chemagro Corp.) to reduce the necessary time of analysis. In all cases, samples were compared with standards that were analyzed within a few hours.

Results and discussion

Organophosphate pesticides were identified in the samples by combining the measurement of retention times on the two columns with the specific response of the flame photometric detector. Three examples of the application of this approach are shown. Methyl parathion, parathion, and DEF were identified by comparing the retention times of peaks in the samples with those of knowns on two columns; the pesticides were confirmed by the presence of peaks at the right retention times with the detector in the sulfur mode. A peak in some samples was shown to be tri-*n*-butyl phosphate, a solvent, rather than phorate by the use of the detector in the sulfur mode.

Differentiation between phorate and tri-n-butyl phosphate. An early peak in a number of samples could have been either phorate or tri-*n*-butyl phosphate from retention times. A typical chromatogram of a sample, recorded with the detector in the phosphorus mode, is shown in Fig. 1a; chromatograms of standards are shown in Fig. 1b and c. Chromatograms obtained on the QF-1 column gave similar agreement

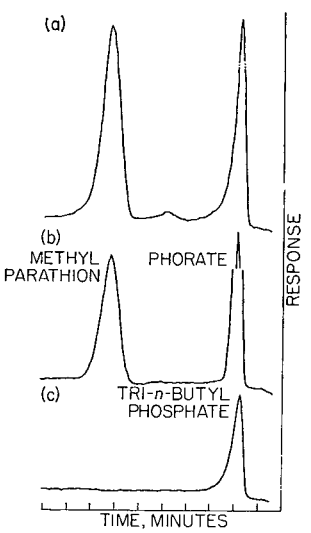

Fig. 1. Chromatograms of pesticides with flame photometric detector in phosphorus mode. (a) Sample, 5.0 μl. (b) Standard containing 4.1 ng each of methyl parathion and phorate. (c) Standard containing 4.2 ng of tri-*n*-butyl phosphate. Column, OV-1; temperature, 150°; amplifier sensitivity, 3.2×10^{-8} a.f.s.

of retention times, except that the retention time of the unknown peak was closer to that of tri-*n*-butyl phosphate than it was to that of phorate. The peak shape, which is often a useful parameter for identifying compounds, more closely resembled the peak for tri-*n*-butyl phosphate than for phorate.

Because the phorate molecule contains two sulfur atoms while tri-*n*-butyl phosphate contains none, the use of the detector in the sulfur mode permitted positive differentiation between the two compounds.

Fig. 2a is a chromatogram of the sample with the detector in the sulfur mode and Fig. 2b is a chromatogram of the standard. The absence of a peak in Fig. 2a at the retention time for phorate shows that the peak in Fig. 1a cannot be phorate but is probably tri-*n*-butyl phosphate.

Identification of methyl parathion. The later peak shown in Fig. 1a was identified as methyl parathion by comparison of its retention times on the two columns with a standard. This identification was confirmed by the use of the detector in the sulfur mode, as shown in Fig. 2.

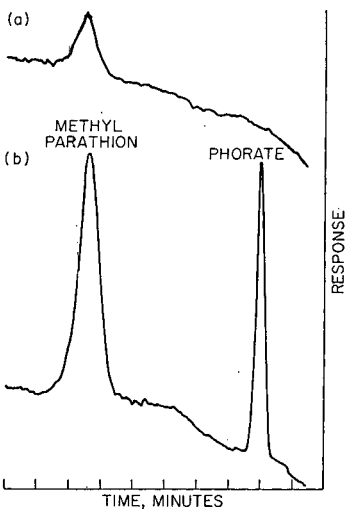

Fig. 2. Chromatograms of pesticides with flame photometric detector in sulfur mode. (a) Sample, 8.5 μl. (b) Standard containing 20 ng of methyl parathion and 4.0 ng of phorate. Column, OV-1; temperature, 150°; amplifier sensitivity, 3.2×10^{-8} a.f.s.

Identification of parathion and of DEF. Parathion and DEF were identified in other samples by a comparison of the retention times on the two columns and confirmed by the use of the detector in the sulfur mode.

Typical chromatograms are shown for a sample containing parathion in Fig. 3. Fig. 3a shows a chromatogram of the sample with the detector in the phosphorus mode; for comparison, a chromatogram of a standard containing parathion is given in Fig. 3b Fig. 3c shows a chromatogram of the sample with the detector in the sulfur mode; for comparison, a chromatogram of a standard containing parathion is given in Fig. 3d. Parathion had the same retention time as malathion on the OV-1 column.

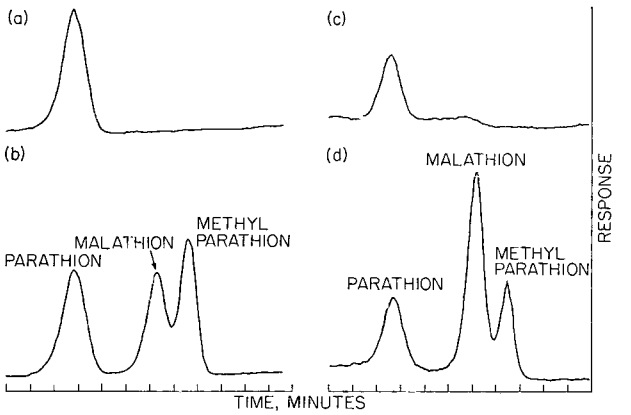

Fig. 3. Identification of parathion. (a) Sample, 5.2 μl; detector in phosphorus mode. (b) Standard containing 5.3 ng each of parathion, malathion and methyl parathion; detector in phosphorus mode. (c) Sample, 14.3 μl; detector in sulfur mode. (d) Standard containing 18.8 ng each of parathion, malathion, methyl parathion; detector in sulfur mode. Column, QF-1; temperature, 140°; amplifier sensitivity, 3.2×10^{-8} a.f.s.

Since the retention times of the sample on both columns agree with those of parathion, the unknown peak was identified as parathion.

Fig. 4a shows a chromatogram of the sample with the detector in the phosphorus mode. Fig. 4b shows a chromatogram of a standard containing DEF with the detector in the phosphorus mode. Fig. 4c shows a chromatogram of the sample with the detector in the sulfur mode. Fig. 4d shows a chromatogram of the standard containing DEF with the detector in the sulfur mode. DEF has essentially the same retention time as malathion on the QF-1 column but has a different retention time on the OV-1 column. The unknown peak was identified by a comparison of the retention times of the peak in the sample with those of a standard on the two columns, and confirmed by the

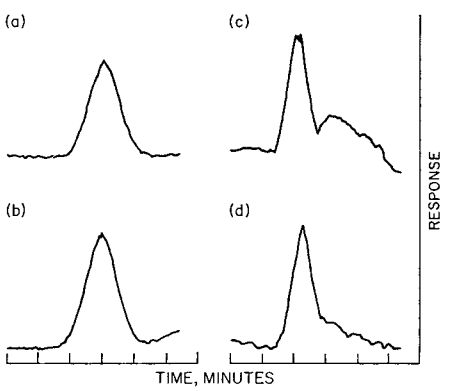

Fig. 4. Identification of DEF. (a) Sample, 5.0 μl; detector in phosphorus mode. (b) Standard containing 5.6 ng of DEF; detector in phosphorus mode. (c) Sample, 16.9 μl; detector in sulfur mode. (d) Standard containing 17.4 ng of DEF; detector in sulfur mode. Column, OV-1; temperature, 158°; amplifier sensitivity, 3.2×10^{-8} a.f.s.

presence of a peak in the sample at the right retention time with the detector in the sulfur mode.

Figs. 3b and d also illustrate the difference in response of the detector in the phosphorus and the sulfur modes. In the phosphorus mode, the detector has roughly equal responses for the three pesticides, methyl parathion, malathion, and parathion; in the sulfur mode, the detector has a greater response for malathion than for methyl parathion or parathion, since malathion contains two sulfur atoms as compared to one sulfur atom in either of the other two pesticides. For compounds which contain one phosphorus and one sulfur atom, at the nanogram level, the response in the phosphorus mode is approximately three times the response in the sulfur mode. Since the response is linear for the detector in the phosphorus mode while it is logarithmic in the sulfur mode[4], all quantitation was done with the detector in the phosphorus mode.

Conclusions

Organophosphate pesticides may be identified by comparing the peak retention times of the unknown sample with those of a known sample on two columns of different polarity and by using the flame photometric detector, which is a specific detector. Since most common organophosphorus pesticides also contain sulfur, the use of the flame photometric detector in the sulfur as well as the phosphorus mode gives further evidence as to the identification.

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CHROM. 3940

Purification of N-trifluoroacetyl amino acid *n*-butyl esters for analysis by gas chromatography

GEHRKE AND STALLING[1] have developed a gas chromatographic method for the analysis of N-trifluoroacetyl *n*-butyl (TAB) derivatives of the 20 amino acids commonly found in proteins. Although all the TAB derivatives may be easily prepared in an impure form, many are oils which cannot be crystallised or are unstable during distillation. The impure preparations frequently contain small amounts of unreacted amino acids which produce multiple peaks during chromatography. In some cases these peaks are eluted slowly and interfere with the resolution of the derivatives of other amino acids. This type of contamination becomes important when radioactive TAB amino acids are eluted and trapped at the end of the column for the measurement of radioactivity, since minor contaminants of high specific activity will obscure TAB amino acids of low radioactivity.

This paper describes a method developed to purify radioactive TAB amino acids using silica gel column chromatography before gas–liquid chromatography (GLC). The incompletely reacted amino acids which are more polar than the TAB amino acids may be separated by elution with organic solvents of various polarities.

Experimental

Silicic acid (British Drug Houses, Poole, England) was soaked overnight in 10 *N* hydrochloric acid, washed with water, ethanol and chloroform and heated overnight at 120°. The silicic acid was then mixed with a light petroleum (b.p. 60–80°) and poured into a 20 × 1 cm column. The TAB derivatives were dissolved in 100–200 μl of dichloromethane and applied to the top of the column, which was then eluted successively with mixtures of 1%, 10% and 50% (v/v) ether in light petroleum, then ether and finally with methanol. Radioactivity in the eluted fractions was measured, using a

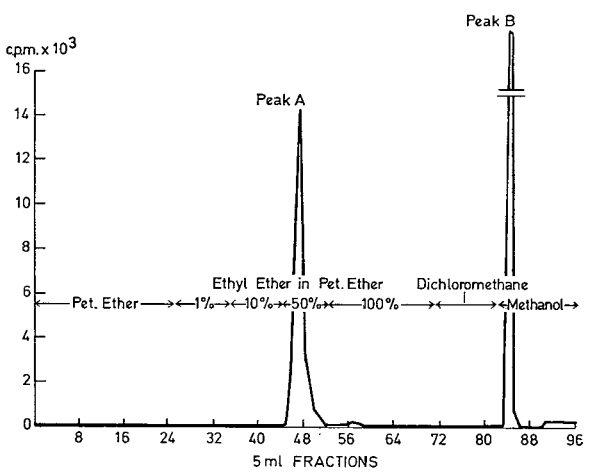

Fig. 1. Distribution of radioactivity in fractions obtained by chromatography on silica gel of the TAB derivative of [U-^{14}C]-L-alanine.

Tri-Carb scintillation counter 314EX, in a toluene phosphor (efficiency 70%) and the radioactive fractions were analysed by GLC. As an example the preparation and the purification of the TAB derivative of [U-^{14}C]-L-alanine is described.

[U-^{14}C]-L-Alanine (sp. act. 2.2 $\times$ 10^6 d.p.m./μmole) was converted to the TAB derivative by the procedure described by GEHRKE AND STALLING[1] and the excess reagents evaporated at a temperature below 60° on a rotary evaporator. The product was fractionated on a silicic acid column by treatment with the solvents indicated (Fig. 1). Samples were analysed for radioactivity and representative fractions were examined by GLC at 140° using the conditions of BLAU AND DARBRE[2].

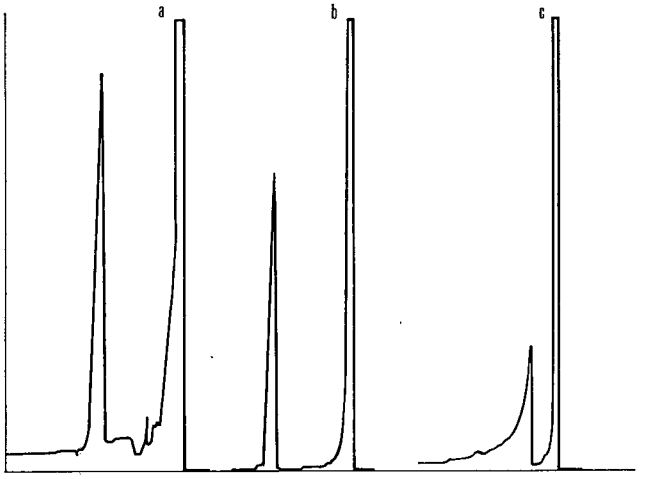

Fig. 2. (a) Gas–liquid chromatography pattern of the unpurified TAB derivative of [U-^{14}C]-L-alanine. (b) Gas–liquid chromatography pattern of the TAB derivative of [U-^{14}C]-L-alanine after purification by silica gel chromatography (this corresponds to peak A in Fig. 1). (c) Gas–liquid chromatography pattern of the methanol eluate (peak B, Fig. 1) by silica gel purification. This behaved similarly to the butyl ester of L-alanine.

Results

Without purification by silica gel chromatography two major and several minor components were detectable by GLC (Fig. 2a). The two major components were separated by silica gel chromatography and gave single radioactive peaks (Figs. 2b and c) in the gas chromatograph, the second compound eluted with methanol from the silica gel column having the same chromatographic behaviour as the *n*-butyl ester of alanine.

Other TAB amino acids may be similarly prepared as well as the *n*-butyl ester of mono- and di-trifluoroacetyl derivatives of the hydroxy amino acids.

Discussion

This method has been used mainly to obtain pure TAB amino acids from impure preparations from tissues and is particularly useful where GLC is used in conjunction with measurements of radioactivity in the GLC eluent since the specific activity of small amounts of purified derivatives can be measured under conditions where quantitation cannot be achieved.

With extracts containing radioactive amino acids and prepared from the livers of mice injected with [U-^{14}C]-glucose the procedure was simplified. The impure mixture of TAB amino acids was applied to the silica gel column, which was washed with 100 ml of light petroleum (b.p. 60–80°), and then the TAB derivatives were eluted with 50 ml of diethyl ether. Also, standard TAB amino acids may become contaminated during preparation and storage but may be readily purified before use by silica gel chromatography.

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CHROM. 3927

Amino acid micro determination with a standard single-column analyzer

In investigations of protein structure the amount of material available for quantitative amino acid analysis and degradation as well as the rate of analysis per day if often a limiting factor. Therefore, it was advantageous to have a single-column system available for the determination of all protein constituent amino acids from one sample, as was described first by PIEZ AND MORRIS[1]. Instruments based on this principle are commercially available (Phoenix Precision Instrument Co., Philadelphia, Pa; Technicon Instrument Co., Ltd., Hanworth Lane, Chertsey, Surrey). Previously, for a complete analysis at least 0.1 μmole of sample had to be applied to the top of a column 0.6 cm (or more) in internal diameter, though only part (30% or less) of the resolved material was used for quantitative detection by the analytical system. BYFIELD[2] described the use of a column 0.3 × 120 cm for the usual 22-hour chromatogram, which permitted sub-micro determination in connection with a voltage amplifier attached to the flow cell colorimeter.

In this paper a similar set of modifications for a standard model of an amino

acid analyzer will be described that enables the apparatus to complete a chromatogram on quantities as low as 0.01 μmole within 11 h without electrical amplification. A column system operating with time switches readily enables two analyses to be carried out daily with no loss of resolution for the protein amino acids. For still greater sensitivity a voltage amplifier may be attached to the colorimeters according to BYFIELD[2].

Methods and materials

The instrument is a standard apparatus (Technicon Auto Analyzer) except that it is equipped with a micro column, 0.3 × 120 cm. The resin is Chromobead ® type C-2, 13 ± 1 μ sperical particles. The developing buffer is pumped by a Milton Roy pump at a volumetric input of 0.21 ml/min, which develops about 350–400 p.s.i. pressure on the column during a run. The column effluent line feeds directly into the nitrogen-segmented ninhydrin stream after the proportioning pump by means of a T piece (Technicon H-3 piece). Therefore all of the resolved material is used for detection. The proportioning pump lines for the nitrogen and ninhydrin stream are approximately one quarter of the usual size, since the column cross-sectional area is a quarter of the usual. The pumping tubes for the proportioning pump are commercially available and deliver 0.16 ml/min of nitrogen and 0.42 ml/min of ninhydrin, while 0.42 ml/min are sucked through the flow cells of the colorimeters by means of a third pump line. Since smaller volumes are delivered into the analytical system, the heating coil for ninhydrin color development had to be shortened to 550–600 cm, giving a heating time of approx. 17 min. All other parts, such as flow cell colorimeters and electrical recording system, remain unchanged. The light path of the longitudinal type flow cells remained 10 mm as usual; however, in the chromatogram shown, the 440-mμ cell had a 5-mm light path. Therefore the proline content of the standard mixture was increased to 0.025 μmole for this chromatogram. The use of a 10-mm light path (or more) is recommended. From the Autograd a modified gradient is supplied with the composition given in Table I.

Results and discussion

The resolution of a typical chromatogram of a standard mixture of 0.02 μmole

TABLE I

GRADIENT (IN ml) FOR 10.5-HOUR CHROMATOGRAM

Chamber	0.05 *M* sodium citrate ($[Na^+]$ = 0.2 *M*)			$[Na^+]$ = 1.2 *M*	$[Na^+]$ = 1.8 *M*
	pH 2.75	*pH 2.875*	*pH 3.80*	*pH 10.00*	*pH 11.50*
1	15.5 2.0 MeOH				
2	13.0	4.5			
3		12.5	5.0		
4			17.5		
5			17.5		
6			11.5	6.0	
7				15.5	2.0
8					17.5
9					17.5

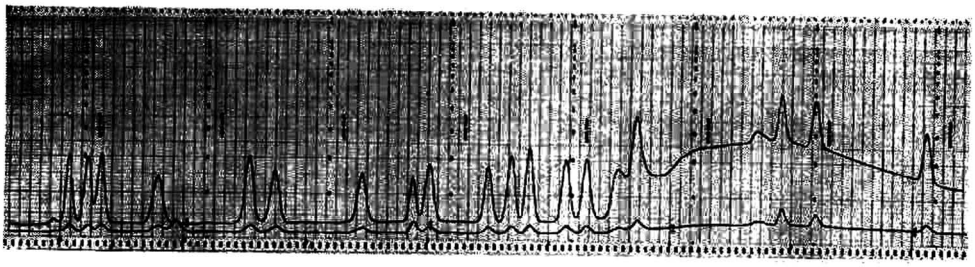

Fig. 1. Typical chromatogram of a standard mixture of 0.02 μmole each of the protein constituent amino acids. Region Asp to Arg. No range expansion. (Pro 0.025 μmole.)

each of the protein constituent amino acids is shown in Fig. 1. A peak representing height is obtained for each amino acid as in the usual chromatogram. The norleucine equivalents differ from the usual quoted values because of the prolonged ninhydrin color development in the heating bath. Repeated blocking of the Teflon sinter at the column outlet due to the narrow particle size range of the resin, could be cured by topping the sinter with a small layer of 1–2 mm of type A resin before packing the column with the Chromobead type C-2 resin. This did not affect the resolution. The column is regenerated by washing with 0.5 *N* sodium hydroxide for at least 1 h, and thereafter it is reequilibrated with a pH 2.875 buffer for as long as is necessary to bring the eluate to the same pH. When not in use, the column is kept under 0.5 *N* sodium hydroxide. This procedure should be followed in order to keep the ammonia baseline down and out of the phenylalanine peak. After the column has been loaded with the sample for analysis, it is filled to the top with the pH 2.875 buffer containing 6% of methanol. The pump lines from the Autograd to the column top are filled with the same buffer (6% in methanol) before they are connected to the Autograd, and the analysis is started. A column prepared in this manner has been in use in our laboratory for more than half a year without repacking. During this time the resin settled about 5 cm, resulting in a slight increase in operating pressure of about 50–100 p.s.i. and extending the time necessary for chromatography by 30–60 min up to 10.5 h. Because the resolution is very sensitive to small changes in buffer pH, buffers have to be carefully stored in a cool place and checked and readjusted if necessary. The reproducibility is ± 5% for duplicates.

It may be concluded that with very little additional equipment an existing apparatus can be altered to gain in sensitivity and to double the number of micro analyses to be carried out daily.

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CHROM. 3924

Desalting of nucleic acid hydrolysates, nucleosides and bases by chromatography on poly-N-vinyl pyrrolidone*

It is often desirable to separate nucleic acid components from salts used in their isolation. Methods available include use of charcoal[1–3], anion exchangers[4,5], cross-linked dextrans[6], polyacrylamide gels[7–9], and extraction with an acetone–ethanol mixture[10,11]. All of these have certain merit, but also possess a number of disadvantages.

We have recently reported on the use of insoluble poly-N-vinyl pyrrolidone for the fractionation of certain nucleotide derivatives[12]. This technique has now been extended and found to be a practical substitute for the above mentioned methods of desalting nucleotides, nucleosides, purines, and pyrimidines. Distilled water is used as eluant, compounds are quantitatively recovered in small volumes, and the bases show clean spectra. No regeneration of the column is required, and it may be used repeatedly for a number of experiments.

Materials and methods

RNA (Na salt, purified from Torula) was obtained from Calbiochem**. DNA (sperm), bases, nucleosides, and nucleotides were purchased from Nutritional Biochemical Co., and were used without further purification. Insoluble PVP (GAF Corporation, New York), sold under the trade name of Polyclar AT Powder, was used in these studies.

The Polyclar AT was mixed with distilled water, and the fines were discarded by repeated decantation. The suspension was poured into a column and allowed to pack with gravity flow. The bed was supported by glass wool.

Hydrolysis of DNA was carried out by heating 20 mg DNA in 1 ml 0.1 *M* H_2SO_4 at 100° for 35 min in a sealed tube. After neutralization with NH_4OH and centrifugation, a 0.5 ml aliquot was added to 0.5 ml 10 *N* LiCl. A 0.3 ml aliquot of this (equivalent to 2.5 mg DNA) was then applied to the PVP column.

RNA was hydrolyzed by heating 4 mg RNA in 0.5 ml 1 *N* HCl at 100° for 1 h. The pH was adjusted to 7 with 1 *N* NaOH and 0.2 ml was applied to the column.

Tests of the separation of bases and nucleosides from ammonium sulfate were

* The following abbreviations will be used: CMP = cytidine monophosphate; UDP = uridine diphosphate; RNA = ribonucleic acid; DNA = deoxyribonucleic acid; PVP = poly-N-vinyl pyrrolidone.

** Mention of trade or company names does not imply endorsement by the Department over others not named.

conducted by addition of standard samples (0.06 mg each of uracil and adenosine) to saturated solutions of ammonium sulfate.

The columns were eluted with distilled water (pH 6) delivered from a one liter container at room temperature and atmospheric pressure. The eluate was collected in 0.5 or 0.6 ml fractions. The effluent volume was determined from the time of sample application and was monitored at 260 mμ in a Gilford 220 spectrophotometer. Chloride

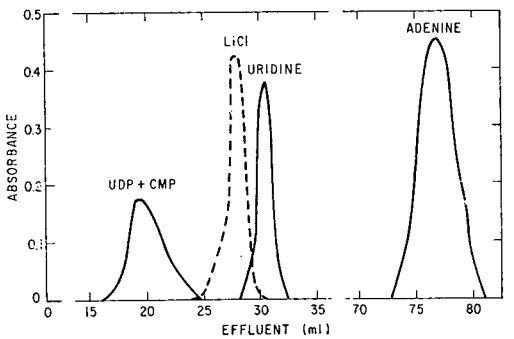

Fig. 1. Chromatogram of an UDP ,CMP, uridine and adenine mixture in 1 *M* LiCl. Column, 1.0 × 39.5 cm; CMP, UDP, adenine (0.04 mg each), uridine (0.024 mg) in 0.2 ml 1 *M* LiCl; eluted with distilled water at a flow rate of 10 ml/h. Absorbance measurements at 260 mμ except for LiCl (as AgCl) at 340 mμ.

was determined with $AgNO_3$–HNO_3 reagent and sulfate with $BaCl_2$ reagent. Both were quantitated by turbidity measurement at 340 mμ. Identification of components was made by comparison with elution volumes of known standards and from the ultraviolet absorbance spectra at neutral, acid and alkaline pH.

Results and discussion

Nucleotide fractions elute prior to NaCl and LiCl; hence, these appear to be excluded from the bed material. Nucleosides and bases exhibit interaction with PVP, resulting in delayed elution. Standard UDP, CMP, uridine, and adenine can be desalted from 1 *M* LiCl on a 1.0 × 39.5 cm PVP column (Fig. 1). NaCl in an RNA hydrolysate

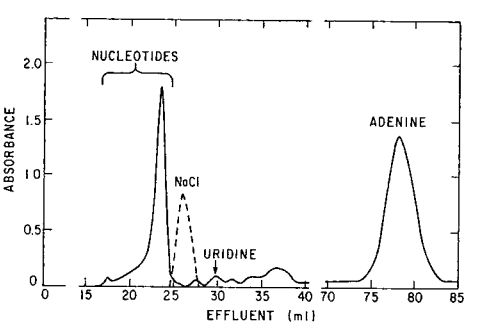

Fig. 2. Fractionation of HCl hydrolysate of RNA. 0.2 ml (equivalent to 0.8 mg RNA) neutralized hydrolysate applied to a 1.0 × 39.5 cm column. Absorbance measurements at 260 mμ except for NaCl (as AgCl) at 340 mμ.

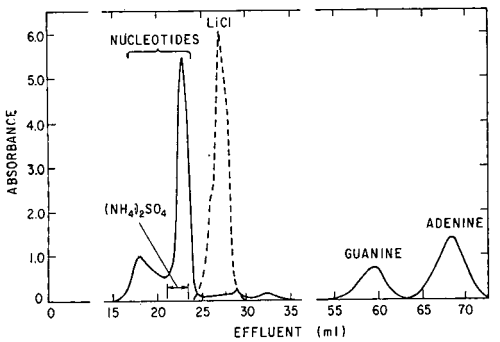

Fig. 3. Chromatography of 0.1 *M* H_2SO_4 hydrolysate of DNA in 5 *M* LiCl. 0.3 ml neutralized hydrolysate (equivalent to 2.5 mg DNA) containing 5 *M* LiCl eluted with distilled water from a 1.0 × 39.5 cm column at a flow rate of 10 ml/h. Absorbance measurements at 260 mμ except for LiCl (as AgCl) at 340 mμ.

behaves similarly (Fig. 2). Fig. 3 shows an H_2SO_4 hydrolysate of 2.5 mg DNA completely desalted from 5 *M* LiCl. Recovery of 260 mμ absorbing material was 99.2%. Hence, pyrimidine nucleotide mixtures derived from ion exchange chromatography of nucleic acid hydrolysates can be desalted and separated from purines in one step.

Desalting of bases and nucleosides on PVP is further illustrated by use of saturated ammonium sulfate. Uracil and adenosine in 0.3 ml saturated $(NH_4)_2SO_4$ can be desalted on a 0.9 × 11.3 cm column in approximately 1 h (Fig. 4). Although some overlap is noted on the short column, complete separation of $(NH_4)_2SO_4$, uracil and adenosine can be attained on a 1.0 × 38.2 cm column. Even in volumes as large as 1 ml of saturated $(NH_4)_2SO_4$ uracil can be completely desalted on such a column. It is evident from Fig. 3 that nucleotides are not separated from $(NH_4)_2SO_4$.

As indicated in a previous publication[12], uracil was eluted from PVP prior to the other bases and nucleosides studied. Since, in the present work, salts elute before uracil, desalting on PVP should be applicable to many bases and nucleosides. Any number of salts should also be separable from bases and nucleosides on PVP, since the

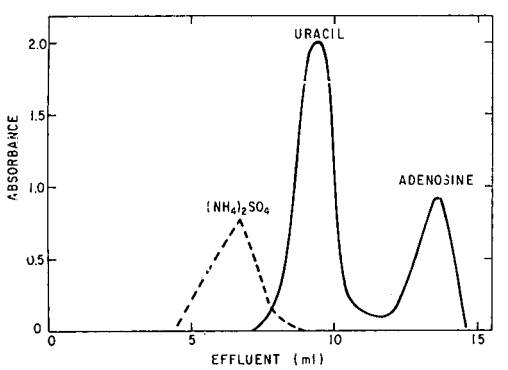

Fig. 4. Fractionation of $(NH_4)_2SO_4$, uracil and adenosine on a 0.9 × 11.3 cm column. Uracil and adenosine (0.06 mg each) in 0.3 ml saturated $(NH_4)_2SO_4$ eluted with distilled water at a flow rate of 14 ml/h. Absorbance measurements at 260 mμ except for $(NH_4)_2SO_4$ (as $BaSO_4$) at 340 mμ.

former apparently filter through the gel without interaction, while the latter are selectively retained.

In cases where slow flow rates present a problem, a bead form of PVP (now under development by GAF Corporation) should be useful.

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CHROM. 3929

Liquid level detector for column adsorption chromatography*

The sensing of liquid levels in fraction collectors is commonly accomplished in a variety of ways such as light absorption, change in refractive index, conductivity, and high frequency oscillator cells. We now describe a very simple, inexpensive, versatile, and trouble-free liquid level detector for the monitoring of eluents in chromatography. This device alerts (by means of a buzzer) the operator when the desired volume of eluent has been collected. This "buzz box" innovation allows the chromatographer to perform other duties while a chromatogram is in progress.

This detector functions by a very simple principle. A schematic for its construction is shown in Fig. 1. The level sensing probe is a thermistor, which is used in a self-heating mode. It senses the presence of eluent by a change in the thermal conductivity of a liquid as compared to the thermal conductivity of the atmosphere. The eluent, when in contact with the probe, conducts more heat from the thermistor, cooling it. This results in a change in resistance of one leg of a balanced Wheatstone bridge circuit. The resultant error signal is amplified by transistors Q_1 and Q_2 to energize the relay R_1. The thermistor used was a glass-coated probe whose two leads were isolated from each other by a piece of small diameter glass tubing. Both leads, housed in a slightly larger diameter glass tube, were then sealed in epoxy cement, after an appropriate length of connecting shielded cable (to amplifier) was attached. The

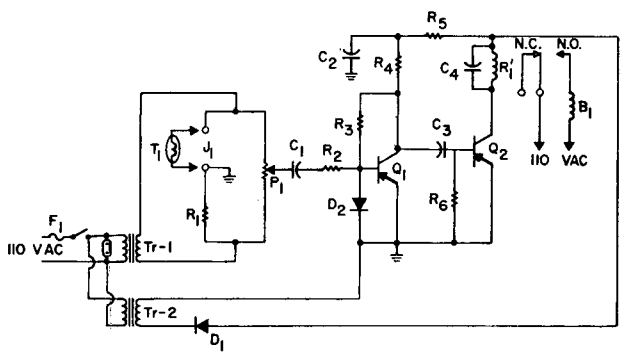

Fig. 1. Schematic for liquid level detector. (T_1) 3kΩ at 25° glass probe thermistor; (P_1) 1kΩ, 2 W; (R_1, R_2) 500 Ω, 1 W; (R_3) 100 kΩ, $^1/_2$ W; (R_4) 5.2 kΩ, $^1/_2$ W; (R_5, R_6) 1.8 kΩ, $^1/_2$ W; (C_1, C_3) 5 MFD, 10 V d.c. electrolytic; (C_2, C_4) 50 MFD, 50 V d.c.; (Q_1, Q_2) 2N1376; (Tr-1) 12 V secondary, 1 A; (Tr-2) 24 V secondary, 1 A; (R'_1) 1500 Ω relay, 2 A contacts; (B_1) 110 V a.c. buzzer; (D_1) IN1692; (D_2) IN60; (J_1) phono jack; (F_1) 1 A fuse.

entire detector was conveniently housed in a portable metal cabinet (*ca.* 15 × 15 × 10 cm) with a panel-mounted fuse box, pilot light, on–off switch, and connector jack to accept the connecting cable from the thermistor probe.

The control of eluent volume was simply determined by the depth to which the thermistor was immersed in the receiving vessel. For our purposes, the thermistor

* Contribution No. 536 of Central Research Laboratories, Minnesota Mining and Manufacturing Company.

was attached (either by a piece of Scotch brand tape or a rubber band) to the drip-tip of the chromatographic column with the precaution that the drops of eluent from the column did not impinge directly on the thermistor (prematurely tripping the buzzer relay).

The normally open relay contacts of the detector were used, in our case, to energize a buzzer. This relay can be employed in a variety of other ways. For example, it may be used as an eluent control by tripping a solenoid-operated plunger valve affixed to the chromatographic column (replacing the stopcock), and thus shutting off the flow of eluent when the desired volume has been collected. In another manner, this device can be positioned to the top of a chromatographic column. It will then warn the operator when the liquid level approaches the stage where the column would go "dry".

With such an easily constructed, inexpensive, and portable instrument, chromatographic operations may become as sophisticated as the operator desires.

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Combination of thin-layer chromatography and the ring-oven method for the semi-quantitative estimation of some herbicides

The importance of the herbicides for the pre- and post-emergence control of weeds has increased in recent years. At present, many of these compounds are officially approved for such use. With the wider application of pesticides in general, the problem of residue determination grows.

The analytical problem presented by pesticide residues in food and other materials has been briefly defined[1–4] and is essentially unchanged. In order to study the relative retention of pesticides (herbicides) in samples of this nature, various methods are required for their microdetermination. MAJOR[5] proposed a paper chromatographic method. ABBOTT *et al.*[6] used a paper chromatographic determination using reflectance densitometry for quantitative measurement, and also thin-layer chromatography[7,8]. The application of the ring-oven technique to herbicide analysis has also been described[9]. Advances in gas chromatographic methods have lately included pesticide residue analysis though the application of gas chromatography to the analysis of these residues was initially confined to chlorinated hydrocarbons and related compounds; however, herbicides are now included[10–12].

The combination of thin-layer chromatography and the ring-oven technique on a semi-quantitative basis was thought to offer a simple, rapid and inexpensive

procedure for detection and determination of minute amounts of some herbicides. The method described in this paper has been found satisfactory in these respects.

Experimental

Five herbicides were studied; they represent different herbicide classes (except two bipyridylium salts) and are listed in Table I.

TABLE I

COMMON AND CHEMICAL NAMES OF THE HERBICIDES USED

Common name	*Chemical name*
Amiben	3-Amino-2,5-dichlorbenzoic acid
CDAA (Randox)	2-Chloro-N,N-diallylacetamide
CIPC	Isopropyl-N-(3-chlorophenyl)carbamate
Diquat	6,7-Dihydrodipyrido[1,2-a:2′,1′-c] = pyrazidinium salt
Paraquat	1,1′-Dimethyl-4,4′-bipyridinium salt

Thin-layer chromatographic separation of herbicides. Thin-layer chromatography has been widely used as method for separation and detection in the study of herbicide residues.

Preliminary experiments were aimed at discovering a suitable procedure for obtaining resonable resolution of the herbicides (*i.e.* separatory-systems), and more sensitive general spraying-reagents for the detection of these compounds on chromatograms.

Chromatoplates of Silica Gel G were prepared (layer thickness: 250 μ) and dried before use in an oven set at 105°. A micro-pipette was used to transfer solutions of the herbicides on to the chromatoplates (Amiben: 0.05%; CDAA: 0.1%; CIPC: 0.1%; Diquat: 0.05%; Paraquat: 0.1%) which were developed by an ascending technique for 45 min or longer (depending on the solvent systems). The plates were allowed to dry and sprayed with suitable spray-reagents.

TABLE II

R_F VALUES OF SOME HERBICIDES IN DIFFERENT SOLVENT SYSTEMS

Compound	*R_F values (× 100)*											
	*1**	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Amiben	0	0	0	0	0	0	0	64	52	—	76	65
CDAA	49	72	74	23	43	69	3	66	86	72	77	72
CIPC	75	81	78	45	58	71	32	82	89	—	—	—
Diquat	0	0	0	0	0	0	0	0	0	25	22	10
Paraquat	0	0	0	0	0	0	0	0	0	26	19	11

* Solvent systems: 1 = chloroform; 2 = chloroform–acetone (9:1, v/v); 3 = chloroform–acetone (8:2, v/v); 4 = hexane–acetone (9:1, v/v); 5 = hexane–acetone (8:2, v/v); 6 = dioxan; 7 = benzene; 8 = benzene–acetic acid glac. (5:1, v/v), 9 = isopropanol–25% NH_3–water (8:1:1, v/v); 10 = isopropanol–HCl conc.–water (5:1:1, v/v); 11 = ethanol–HCl conc.–water (6:1:3, v/v); 12 = methanol–HCl conc.–water (6:1:3, v/v).

Table II shows the effect of different developing systems on the thin-layer chromatographic separation of the herbicides.

A combination of $NaNO_2$ and N-1-naphthyl-ethylenediaminedihydrochloride has been successfully used for detecting Amiben spots[13]. Aqueous 0.5% $KMnO_4$ was used for CDAA and also for Amiben visualisation, a combination of 0.5% $AgNO_3$ (acidified with 2 ml of HNO_3) and 0.5% $KMnO_4$ was used for CIPC and methanolic NaOH (followed by heating in a stream of warm air) for Diquat and Paraquat visualisation. Table III lists the observed practical sensitivities obtained on developed chromatoplates for each of the compounds studied.

Semi-quantitative determination of separated herbicides using the ring-oven technique. The herbicides were determined individually with the Weisz ring-oven by spotting aliquots of the stock solutions of the appropriate herbicide on to the 5.5 cm diameter filter paper (Whatman No. 1) by means of a 10 μl micro-pipette. The substances were then washed into ring-zones as follows: Amiben with 0.5 ml of water, CDAA and CIPC with 0.5 ml of acetone, Diquat and Paraquat with 0.5 ml of 3 *N* HCl. The paper was dried in a stream of warm air (except when acetone is used).

The spray-reagents mentioned above were also used successfully for the detection of the ring-zones; the practical sensitivities being listed in Table III.

The method used for the semi-quantitative determination has already been reported[9,14]. The amount of herbicide (C_a) present can be expressed as:

$$C_a = C_s \frac{V_s}{V_a}$$

where C_s is the concentration of the standard solution, V_a the volume of sample solution and V_s the volume of standard solution used in the matching ring.

Combination of the thin-layer chromatography and ring-oven techniques. From a survey of the literature it is apparent that thin-layer chromatography has rapidly developed into a precise technique of great value to the pesticide residue analyst, but the need to find, identify and determine a very small amount of an organic compound in the presence of large quantities of natural materials can be extremely difficult and sometimes quantitative evaluation of chromatograms, based upon the measurement of spot area, will be impossible. The final determination step and conclusions can easily be carried out in such cases by means of a combination of thin-layer chromatography and the ring-oven technique.

TABLE III

THE SENSITIVITY OF HERBICIDES TO SPRAY-REAGENTS

Compound	*Sensitivity (µg)*	
	*TLC**	*ROT***
Amiben	0.25	0.10
CDAA	0.75	0.75
CIPC	0.50	1.00
Diquat	0.50	0.50
Paraquat	1.00	1.00

* Thin-layer chromatography.
** Ring-oven technique.

After thin-layer chromatography of the herbicide solutions, the separated herbicides were scraped from the chromatoplates quantitatively into a funnel (porosity G 4), mounted above the oven. Each batch of scrapings was washed with 1 ml of the appropriate solvent on to the 5.5 cm diameter filter paper. The substances were then washed into ring-zones and detected as explained above. Stable red rings for Amiben (over 30 days) were obtained on a white background; stable brown rings for Diquat and bluish-green rings for Paraquat were obtained on a white background. Unstable yellow rings (20 min) on a pinkish background were obtained for CDAA and CIPC.

Conclusions

The intrinsic inexpensiveness of the apparatus and the simplicity, speed and versatility of the technique described make it auspicious for the diagnostic, semi-quantitative and preparative studies of herbicides and their residues in contaminated samples.

Minute amounts of herbicides could be isolated and detected on the thin-layer chromatoplates, and quantitatively transfered to the ring-oven for determination.

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TAS-method for the microanalysis of important constituents of saffron

The principal pigments of Spanish saffron are the bitter-tasting picrocrocin, a 4-[glucopyranosido]-2,6,6-trimethyl-1-cyclohexene-1-aldehyde and the water-soluble orange-red carotenoid crocin, a digentiobioside of the aglycone crocetin, a heptaenedicarboxylic acid.

During detailed investigations[1] it was discovered that a "thermomicro separation" (by the so-called TAS-method; refs. 2–4) of the components is quite possible, and some new aspects of common interest were demonstrated.

At a temperature of 200° and a reaction time of 1 min, a definite fission of picrocrocin occurred, and the free aglycone, a 2,6,6-trimethyl-1,3-cyclohexadiene-1-aldehyde designated safranal, appeared immediately on the TLC plate. The orange-red carotenoids, however, remained in the stigma. After treating some milligrams of powdered saffron stigmas with an ethereal solution of diazomethane, fission of crocin occurred followed by methylation to the *trans*-dimethyl ester of crocetin, as shown by TLC. Using redried saffron powder in the TAS-oven at 200°, the compound described appeared on the plate as a yellow spot. The separation was accomplished by TLC on Silica Gel HF_{254} using benzene–chloroform (80 + 20) under standard conditions. Under shortwave U.V. light a fluorescence-quenching spot appears at $hR_F = 60$, and a yellow spot was visible in daylight at $hR_F = 45$.

For further identification, the spectra were measured directly on the plate[5] (max. 240 and 280 nm = safranal and max. 456 and 480 nm = *trans*-dimethyl ester of crocetin). After the spectrophotometric determination the chromatogram was treated with antimony trichloride reagent. Even at low temperatures the safranal zone shows a yellow color which becomes green-yellow after heating for 10 min at 110°. The carotenoid undergoes the Carr–Price reaction turning blue.

The experiment described shows that the combination of the TAS-method with TLC is very useful for the indication of the characteristic constituents of saffron. This can be significant in view of the numerous adulterations. Further experiments with other β-glucosidic compounds not mentioned here showed that they can also be broken down in the same manner.

In addition to the esterification and cleavage, the synthesis of trimethylsilyl ethers can also be useful.

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CHROM. 3944

Effects of drying and equilibration of paper chromatograms of tritium labelled compounds

Experiments with ^{14}C-labelled compounds have shown that unequal drying of the two surfaces of a paper chromatogram may lead to unequal distribution of the solute throughout the thickness of the paper, both at the time of application[1,2] and after development[3]. This effect is more readily detected when ^{3}H is used as a tracer owing to the very soft β-emission of this isotope (maximum 18.5 keV) which has a range of only 0.006 mm in a medium of unit density compared with the range of 0.2 mm of ^{14}C; and we have found that chromatograms from which emission from one side of the paper is five times that from the other can be readily prepared.

25 μl of an aqueous solution of D-glucose-3-^{3}H (0.5 mC/ml, 77.6 μg/ml) was applied as one lot to Whatman No. 1 paper and immediately dried by a hot air blower directed perpendicularly to the front (top) face of the paper which was suspended horizontally. Each side of the spot was then scanned with a windowless gas flow proportional scanner and the paper developed in *n*-butanol–ethanol–water (104:66:30). The paper was then dried as above and again scanned. Control chromatograms were also prepared. These were dried in still air at room temperature both after application

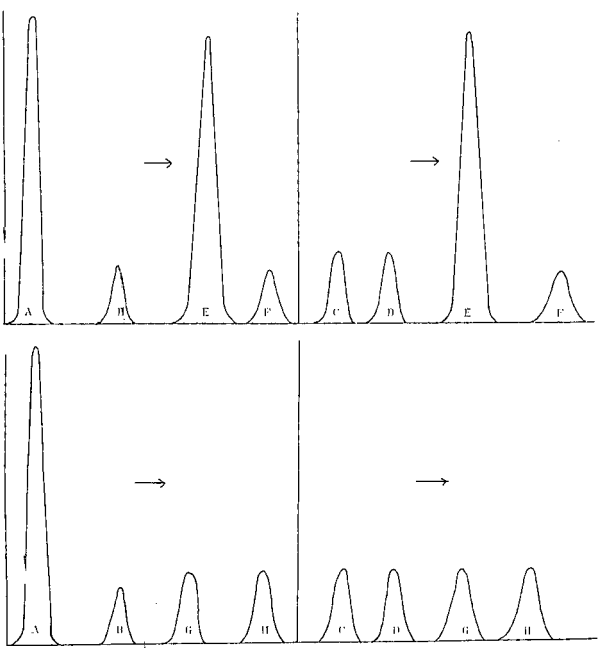

Fig. 1. Scans of peaks of paper chromatograms, D-glucose-3-^{3}H or L-leucine-4,5-^{3}H. (A) Front of paper dried with hot air blower before development, (B) back. (C) Front of paper dried in still air before development, (D) back. (E) Front of paper dried with hot air blower after development, (F) back. (G) Front of paper dried in still air after development, (H) back.

of the solute and after development. An aqueous solution of L-leucine-4,5-^{3}H (0.5 mC/ml, 2.9 μg/ml) was treated similarly. The results are summarized in Fig. 1 and clearly confirm the observations of DUNCOMBE[3] that the type of drying after development is the major factor determining the shape of the scan, since solute unequally distributed at the origin (A, B) is essentially evenly distributed at each surface of the paper (G, H) after development and remains so provided that uniform drying is applied to the paper. This behaviour should be general for water-soluble compounds developed with aqueous solutions, but compounds sparingly soluble in water do not always become evenly distributed during development. For example, DOBBS[4] has shown that benzoic acid and stearic acid having different concentrations on opposite sides of the same paper maintained this difference after a chromatogram had been run in ethanol–0.880 ammonia–water (16:1:3). We have on occasion observed very variable results when paper chromatograms of ^{3}H labelled compounds are dried in a fume cabinet and we suggest that drying in a draught free room is essential for the attainment of reproducible results.

We were recently informed[5] that the radioactivity of some ^{3}H labelled steroids supplied by us "disappeared" during paper chromatography under certain conditions. There was no evidence that the tritium in these compounds was labile or volatile, and we now put forward two possible explanations for this observation. It appears that the steroids are either concentrated at the surfaces of the paper at the time of application and are then essentially evenly distributed throughout the thickness of the paper after equilibration and/or development, or they are essentially evenly distributed on application and became concentrated at the centre of the thickness of the paper after equilibration. It is possible that microautoradiography of a section through the paper, as suggested by HAIS (see ref. 2), could determine which explanation is the more correct. We prefer the former explanation since the steroids had a lower R_F in the equilibrating solvent than in the solvent used to apply them (see below) and DUNCOMBE[3] reports that compounds with high R_F values tend to be more readily concentrated at the surface of the paper during drying. 15 μC aliquots of a benzene solution of progesterone-7α-^{3}H (2 mC/ml, 74 μg/ml) or oestrone-6, 7-^{3}H (1 mC/ml, 7.3 μg/ml) were applied to Whatman No. 1 paper as a line 4 cm long, and "run up" to a predetermined origin line according to the method of BUSH[6]. The papers were equilibrated for 18 h in a tank containing petroleum ether (b.p. 60–80°) and 90% aqueous methanol, and developed with petroleum ether (b.p. 60–80°). The papers were dried in still air after application of the spot, running up, and development. One paper of each steroid was removed from the tank and dried in still air after the equilibration stage. Scans of the chromatograms were made at each stage and are shown in Fig. 2. Scans of either side of each paper were virtually identical. The apparent loss of radioactivity at the equilibration stage was substantial and a decrease in peak area of about 90% was usual. After development, the area of paper containing the steroid was cut out and combusted to water using a Schöniger technique, and the water assayed by liquid scintillation counting in a scintillant of naphthalene (180 g), PPO (10 g), POPOP (0.1 g) in 2 l of xylene–dioxan–water (1:1:1). Recoveries of at least 14.5 μC tritium were obtained, the small loss of radioactivity being probably caused by "streaking" or irreversible adsorption at the origin of the chromatogram since no carrier steroid was added. We then spotted 0.25 μC samples of each steroid onto paper and assayed the spots before or after equilibration as above by adding the radioactive area of the

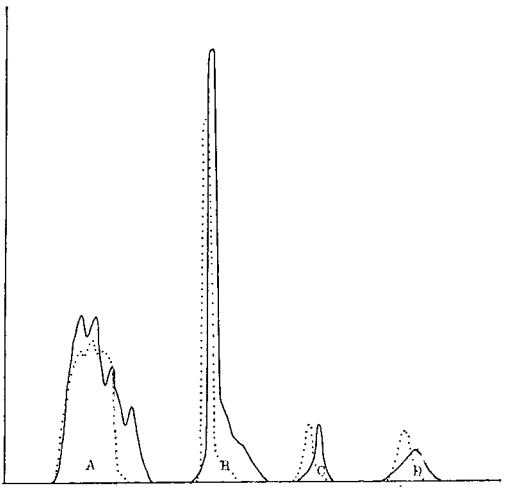

Fig. 2. Scans of peaks of paper chromatograms, (———) progesterone-7α-^{3}H, (· · · · ·) oestrone-6,7-^{3}H. (A) Origin, (B) after running up, (C) after equilibration, (D) after development.

paper directly to a scintillant of PPO (0.5 g) POPOP (0.01 g) in 100 ml of toluene. By this method about 80% of the tritium was detected before equilibration but only 20% after equilibration. Since this result appears to confirm that of the scanning, it is important to realise that absorption of the β-emission is again the cause of the spurious low result. This problem of self-absorption and other complications involved in the direct liquid scintillation counting of tritium labelled chromatograms has been reviewed by TURNER[7] and by DE BERSAQUES[8], and, as we have never been able to obtain satisfactory results in our laboratories by this method, we recommend that the combustion technique is used whenever possible.

If the direct method must be used we conclude that, in view of the effects of unequal distribution of the solution discussed above, complete solution of the solute into the scintillant is desirable if quantitative results are required.

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Artefacts in the paper chromatography of D-mannosamine-1-^{14}C hydrochloride

In the course of the analysis of D-mannosamine-1-^{14}C hydrochloride synthesized at the Radiochemical Centre artefacts were detected in the paper chromatographic procedures used as a direct result of the sensitivity of detection by the radioactive method.

D-Mannosamine-1-^{14}C hydrochloride is prepared essentially as the method of KUHN *et al.*[1]. The reaction results in two epimers, D-glucosamine-1-^{14}C hydrochloride and D-mannosamine-1-^{14}C hydrochloride, in the ratio roughly of 4 to 1. These are separated by preparative paper chromatography. The resultant purified material is crystallised from moist methanol by the addition of acetone and dried *in vacuo*.

Several artefacts, notably double-spotting and streaking, have been reported in the paper chromatography of amino sugars[2–4], and low loadings and strongly basic solvent systems have been recommended to counteract them, but these lead to decomposition including the formation of glycosylamines[5].

This note reports the occurrence of artefacts in the paper chromatography of radioactive materials even in the recommended systems. The apparently low radiochemical purity values found are shown to be due to these artefacts, which are undetectable in the paper chromatography of inactive material. The detection of artefacts by conventional (inactive) colour methods depends on the sensitivity of the reagent and this will probably be two or three orders of magnitude less sensitive than radioactive methods.

This compound provides yet another example in which the ideal of a symmetrical "spot" is not attained in any of the systems tried; "streaking" always occurs, even with what is believed to be a pure compound, and with care taken to avoid overloading or underloading of material on the paper.

Materials and methods

D-Mannosamine-1-^{14}C hydrochloride had a specific activity of 45 mC/mmole. Carrier D-mannosamine hydrochloride was purchased from Sigma Chemical Company Ltd., London and was recrystallized from water–methanol–acetone. It had m.p. 178–178.5° (d) $[\alpha]_D^{20}$ — 3.2° (C.1 in water); literature values[6] m.p. 178–180°, $[\alpha]_D^{20}$ —3.0°.

Purified diluted samples in the reverse isotope dilution analysis were obtained by recrystallization to constant specific activity. The samples were dissolved in water and counted by liquid scintillation methods using a Nuclear Chicago Mk. I counter and Triton X-100 scintillant[7]. Counting efficiency was determined by the Channels Ratio method and suitably predetermined background counts were subtracted from all sample counts.

Standard solutions were applied to the chromatograms from calibrated micropipettes and allowed to dry at room temperature. The papers used were all Whatman types and are described in the Tables I and II. Four solvent systems were used for development:

(A) *n*-butanol–ethanol–water(52:33:15),

(B) pyridine–ethyl acetate–acetic acid–water (5:5:1:3),

TABLE I

RADIOCHEMICAL PURITY VALUES DETERMINED IN SOLVENT SYSTEMS A, B, C, D FOR D-MANNOSAMINE-1-^{14}C HYDROCHLORIDE

Chromatogram No.	*Solvent system*	*Paper type*	*Loading (μg)*	*R.C. purity (%)*	*Streaking*
1	A	No. 1	13**	93	+
2	A	No. 1	10	93	+
3	A	No. 1	3	93	+
4	A	No. 1	0.5	93	+
5	A	No. 541*	3	<85	++
6	B	No. 1	13**	95	+
7	B	No. 1	10	96	+
8	B	No. 1	3	96	+
9	B	No. 1	0.5	96	+
10	B	No. 541*	3	<90	++
11	C	No. 1	13**	97	+
12	C	No. 1	10	97	+
13	C	No. 1	3	96	+
14	C	No. 1	0.5	97	+
15	C	No. 541*	3	<92	++
16	D	No. 1	3	95	+

* Acid-washed paper.
** 3 μg active material + 10 μg carrier.

TABLE II

COLOUR YIELDS AND STREAKING OF D-MANNOSAMINE HYDROCHLORIDE IN SOLVENT SYSTEMS A, B, C; WHATMAN NO. 1 PAPER

Solvent	*Loading (μg)*	*Colour intensity of spot**	*Streaking*
A	1	J.V.	–
A	5	L	–
A	10	M	–
A	20	H	–
A	50	H	+
A	100	H	++
B	1	J.V.	–
B	5	L	–
B	10	M	–
B	20	H	–
B	50	H	+
B	100	H	++
C	1	N.D.	–
C	5	J.V.	–
C	10	L	–
C	20	M	–
C	50	H	+
C	100	H	++

* N.D. = not detectable; J.V. = just visible; L = light; M = medium; H = heavy.

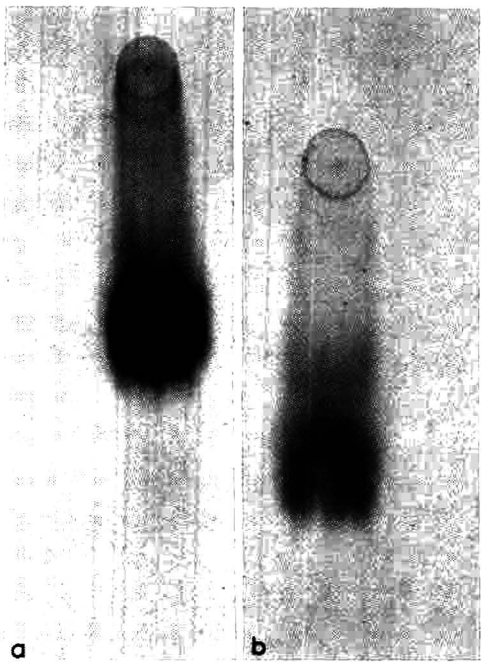

Fig. 1. Autoradiographs of paper chromatograms of D-mannosamine-1-^{14}C-hydrochloride; (a) First run, (b) rerun of active spot eluted from (a). Solvent system A, Whatman No. 1 paper.

(C) ethyl acetate–acetic acid–water (9:2:2), containing 2% phenylboronic acid,

(D) system (A) containing 0.15% w/v ethylenediamine tetraacetic acid, sodium salt.

After development the chromatograms were dried in air at room temperature and the inactive papers sprayed with a 0.5% solution of ninhydrin in *n*-butanol. They were then heated in an oven at 100° for 5 min and the resulting reddish-purple spots noted.

Active chromatograms were scanned to check peak symmetry. They were autoradiographed on "Kodirex" X-ray film and the active areas marked out, cut out and placed into counting vials containing toluene–PPO liquid scintillant. These were counted by liquid scintillation methods. The activity in the major spot was then expressed as a proportion of the total activity along the solvent track.

Results and discussion

Duplicate reverse isotope dilution analysis with D-mannosamine hydrochloride carrier gave a mean radiochemical purity value of 99%. Reverse isotope dilution analysis with D-glucosamine hydrochloride carrier gave a glucosamine content of 2%. Radiochemical purity values from paper chromatography in all four systems are given in Table I.

From the results of the dilution analyses it would appear that the D-mannosamine-1-^{14}C hydrochloride has a radiochemical purity value in the region of 98%. However, paper chromatography systems give consistently low apparent radio-

chemical purities due to heavy streaking of the material on the paper. These purity values are not affected significantly by the use of ethylenediaminetetraacetic acid in the solvent, nor by changing the loading of active material, nor by a fourfold diminution in the specific activity of the active material by dilution with carrier D-mannosamine hydrochloride. The streaking is greatly aggravated by the use of acid-washed papers.

When the principal radioactive spot from chromatogram No. (2) was eluted off and re-run in solvent (A), it gave a radiochemical purity value of 90%, with the same pattern of streaking (see Fig. 1). This was the fourth successive time the material had been chromatographed in similar systems (twice preparatively and twice analytically) and yet it still showed activity streaking back to the origin.

Inactive carrier D-mannosamine hydrochloride was chromatographed with different loadings of material in three systems, as shown in Table II.

It is evident that streaking only becomes apparent with ninhydrin in paper chromatography systems when the loading of material is in the region of 50 μg. But 1 μg of amino sugar (in a spot of normal size) is the threshold of detection by normal colour methods. Hence it is quite possible for streaking to occur at loadings of less than 50 μg and yet for it to be quite undetectable by colour. For example, 20 μg loading with 5% streaking would give 1 μg streaked over a paper area corresponding to, say, ten times the normal spot area.

It is to be noted that neither a fourfold diminution in the specific activity of the active material nor the use of an alkaline solvent (system B) significantly affects the streaking and hence the origin of streaking is only partly due to irreversible absorption onto the paper by an attraction between the mannosammonium ion and the carboxylate ions present in the paper.

CATCH has described several artefacts which can give misleading results in the paper chromatography of radioactive compounds[8]. To these must be added those due to the selection of the solvent system used, since although it may apparently be suitable for inactive materials it may lead to artefacts which can be detected only by the very much more sensitive radioactive methods. The above evidence shows that D-mannosamine-1-^{14}C hydrochloride is a compound which inherently gives rise to streaking.

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CHROM. 3941

Separation of the diastereoisomers of 3-hydroxyglutamic acid by ion-exchange chromatography

As a preliminary to a study of the metabolism of 3-hydroxyproline, we needed a chromatographic method for the separation of the diastereoisomers of 3-hydroxyglutamic acid. Ion-exchange chromatography has been used for the separation of the diastereoisomers of several amino acids with two asymmetric carbon atoms (for a review, see ref. 1). No separation of the diastereoisomers of 3-hydroxyglutamic acid occurred under those conditions in which there was complete separation between the diastereoisomers of 4-hydroxyglutamic acid and of 3-hydroxyaspartic acid[2–4], and another procedure was therefore worked out.

Materials and methods

DL-DL-*allo*-3-Hydroxyglutamic acid was obtained from Fluka AG, Buchs, Switzerland. Dowex AG 50W-X8 (200–400 mesh, hydrogen form) was obtained from BioRad Laboratories, Inc., Richmond, Calif., U.S.A. Acetone-dried cells of *E. coli* and *Cl. welchii* were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. and Worthington Chemical Corp., Freehold, N.J., U.S.A.

The quantitative determination of the amino acids was performed according to MOORE AND STEIN[5]. The manometric determination of decarboxylase activity in 0.2 *M* sodium acetate buffer, pH 5.0, was carried out by the Warburg technique. Ion-exchange chromatography was carried out in glass columns at room temperature; 0.07 *M* HCl was used as eluting agent, and the rate of elution was 0.15 ml per min per cm^2. Fractions were collected by means of an automatic fraction collector with a drop counter. Paper chromatography (descending) was carried out with pyridine–water (4:1) as described by UMBREIT AND HENEAGE[6].

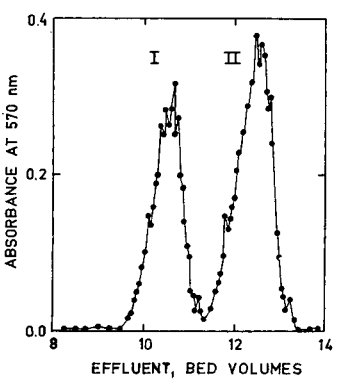

Fig. 1. Chromatography on a column of Dowex AG 50W-X8 (200–400 mesh, hydrogen form, 3.0 × 56 cm) of 600 mg of DL-DL-*allo*-3-hydroxyglutamic acid with 0.07 *M* HCl. The concentration of amino acid was determined by means of the ninhydrin reaction according to MOORE AND STEIN[5].

TABLE I

RATES OF DECARBOXYLATION OF THE MATERIAL IN PEAK I AND PEAK II WITH DIFFERENT SOURCES OF ENZYME

The incubations were carried out at 37° in Warburg vessels with 0.2 *M* sodium acetate buffer (pH 5.0), 10–30 mg of acetone-dried cells and an initial concentration of 0.010 *M* substrate in a total volume of 2 to 3 ml.

*Enzyme (batch)**	*Initial rate of CO_2 evolution, μl CO_2 per min per mg of enzyme*		*Ratio I/II*
	Peak I	*Peak II*	
E. coli (Sigma, type I, batch No. 73B-8110)	0.54	0.056	9.6
E. coli (Sigma, type II, batch No. 75B-9160)	2.58	0.32	8.1
E. coli (Worthington, GLD 6442)	2.3	0.32	7.2
Cl. welchii (Sigma, type II, batch No. 43B-647)	0.23	0.032	7.2

* Acetone-dried cells.

Results

Fig. 1 shows the separation of the diastereoisomers of 3-hydroxyglutamic acid on a cation exchanger with dilute hydrochloric acid as eluting agent. The commercial preparations of 3-hydroxyglutamic acid have been found to contain various amounts of 4-amino-3-hydroxybutyric acid, which, however, was eluted far more slowly than the dicarboxylic acid under these conditions. The material in each peak (peaks I and II, Fig. 1) was rechromatographed, taken to dryness and recrystallized from hot ethanol. The melting points for the hydrochlorides were: I, 194–195°; II, 190–192°. These materials were treated with different batches of commercial preparations of acetone-dried cells of *E. coli* and *Cl. welchii*. As shown in Table I, the material in peak I was decarboxylated at about an 8-fold higher rate than that in peak II, when such concentrations were used that the initial rate of decarboxylation was independent of the substrate concentration. In separate experiments it was ascertained that there was no gas uptake or release when 4-amino-3-hydroxybutyrate was incubated with the acetone-dried cells, either with or without potassium hydroxide in the center well. About 46% of the material in peak I was decarboxylated, as was about 41% of that in peak II. 4-Amino-3-hydroxybutyric acid could be demonstrated as the reaction product, using chromatography on Dowex 50 and on paper.

Discussion

The diastereoisomers of 3-hydroxyglutamic acid have previously been separated by fractional crystallization of the hydrochlorides of the free amino acid or its diethyl ester of the N-acetyl diethyl ester derivatives[7,8]. The *allo*-form[7] (*threo*-form[9]) is decarboxylated at a severalfold higher rate[6,8,9] than the *normal* form (*erythro*-form), and KANEKO AND YOSHIDA[10] have demonstrated the formation of (−)-4-amino-3-hydroxybutyric acid as the reaction product of the decarboxylation of the *threo*-form. This compound has the same configuration as the natural form of carnitine[10]. The difference in the susceptibility to enzymic decarboxylation between the diastereoisomers of 3-hydroxyglutamic acid is similar to that found with the diastereoisomers

of 5-hydroxylysine[11]. The ratio between the initial rates of decarboxylation of the two diastereoisomers of 3-hydroxyglutamic acid was of the same order of magnitude as that reported by other authors[6,8,9], and the material in peak I (Fig. 1) may therefore be designated as *threo*-3-hydroxy-DL-glutamic acid, and that in peak II as *erythro*-3-hydroxy-DL-glutamic acid.

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CHROM. 3925

Reproduzierbare Gradientenchromatographie an Kolonnen mit schrumpfenden Austauschern

Die Methoden der chromatographischen Fraktionierung von Proteingemischen oder solchen anderer hochmolekularer Naturstoffe haben seit Einführung passend substituierter Materialien mit Cellulose-[1] oder Dextrangerüst als Ionenaustauscher eine weltweite Anwendung gefunden. Die technische Weiterentwicklung derartiger Austauscher führte zu Produkten, welche auch ohne Anwendung von Druck befriedigende Durchflussgeschwindigkeiten während der Chromatographie liefern.

Bei steigender Ionenstärke des Elutionsmittels zeigen diese Austauscher indessen eine mehr oder minder ausgeprägte Schrumpfung ihres Volumens, die bei starkem Anstieg der Ionenstärke während eines Chromatographieprozesses 40–60% des ursprünglichen Bettvolumens ausmachen kann. Während Fraktionierungen mit stufenweiser Elution durch ein derartiges Verhalten des Gel-Bettes nicht wesentlich beeinträchtigt werden, muss eine Gradientenchromatographie durch eine solche Volumenverminderung des Austauschers empfindlich gestört werden: Oberhalb des

sich kontrahierenden Gel-Bettes bildet sich eine zusätzliche "Mischkammer", in welcher das Eluens, bevor es in die Kolonne einsinkt, verdünnt wird, was zu einer unkontrollierbaren Verflachung des Gradienten Anlass gibt. Da bei Verwendung von Kolonnen unterschiedlicher Dimensionen oder solchen mit verschiedener Füllhöhe die "Mischkammer" auch verschiedenes Volumen besitzt, lassen sich vergleichende Untersuchungen über das chromatographische Verhalten von Substanzgemischen trotz Verwendung desselben Gradienten nicht reproduzierbar durchführen.

Die erwähnten Schwierigkeiten würden entfallen, wenn man das sich in seiner Konzentration ständig ändernde Elutionsmittel direkt auf die Oberfläche des schrumpfenden Gels in kleinen Volumenfraktionen so aufbringen könnte, dass es nicht zu einem störenden Überstand von Puffer auf dem Austauscherbett kommen kann.

Eine Anordnung, welche diese Anforderungen erfüllt und sich seit mehr als zwei Jahren in unserem Laboratorium besonders beim Arbeiten mit DEAE-Sephadex A 50* bewährt hat, wird nachstehend beschrieben.

Der Apparat besteht aus zwei Teilen, einem Adapter (Fig. 1), welcher direkt auf dem Austauscherbett aufsitzt und einer Steuereinheit (Fig. 2), die für den passenden Zufluss von Elutionsmittel sorgt. Die Funktion des Gerätes lässt sich folgendermassen beschreiben: Eine Pumpe fördert Elutionsmittel, welches über den Adapter der Austauscheroberfläche (Fig. 1b) zugeführt wird. Der ansteigende Flüssigkeitsspiegel (Fig. 1c) komprimiert das in der Luftkammer des Adapters eingeschlossene Luft-

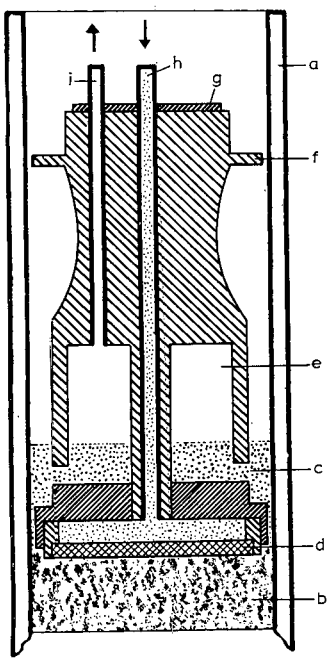

Fig. 1. Adapter. (a) Chromatographiekolonne; (b) Austauscher; (c) Elutionsflüssigkeit; (d) Fritte; (e) Luftkammer; (f) Abstandshalter; (g) Bleischeibe zur Gewichtskorrektur; (h) Zuführungsrohr für die Elutionsflüssigkeit; (i) Luftkammerrohr.

* Deutsche Pharmacia GmbH, Frankfurt/M.

volumen. Diese Drucksteigerung wird über einen feinen Schlauch einem Druckwellenschalter zugeleitet, der beim Ansprechen seiner Membran die Pumpe abschaltet und damit den Flüssigkeitszufluss unterbricht. Nach Ablauf einer an einem Relais einstellbaren und von der Chromatographiergeschwindigkeit abhängigen Zeit, während welcher das Eluens in das Gel-Bett eingesunken ist, wird die Pumpe wieder in Betrieb gesetzt und der Prozess wiederholt. Es gelingt auf diese Weise, das Elutionsmittel in kleinen Anteilen von *ca.* 1–2‰ des Gesamtvolumens des Gradienten der Kolonne zuzuführen.

Der Adapter (Fig. 1) ist aus Plexiglas hergestellt und besitzt an seiner breitesten Stelle einen um *ca.* 1 mm kleineren Durchmesser als das verwendete Chromatographierohr (Fig. 1a). Der Boden des Adapters enthält eine Vylon-1-fritte (Fig. 1d), durch welche das Elutionsmittel (Fig. 1c) auf das Gel-Bett gelangt. Das Gewicht des Adapters ist so bemessen, dass er dem schrumpfenden Gel folgen kann ohne jedoch in es einzusinken. Für Kolonnen mit 33 mm Durchmesser hat sich ein Gewicht von *ca.* 20 g bewährt, für solche mit 16 mm eines von *ca.* 11 g. Durch Auflegen von flachen Bleischeiben (Fig. 1g) kann man das Gewicht des Adapters variieren und den geforderten Verhältnissen anpassen. Ein Abstandshalter (Fig. 1f) verhindert ein Verkanten oder Festklemmen des Adapters im Chromatographierohr. Ausser dem Zuführungsrohr für den Puffer (Fig. 1h) (I.D. 1 mm) befindet sich exzentrisch dazu ein ebenfalls aus PVC bestehendes Rohr (Fig. 1i), welches die Luftkammer (Fig. 1e) über einen dünnen Silikonschlauch (I.D. 0.8 mm) mit dem Druckwellenschalter der Steuereinheit verbindet. Die Kompression des in der Kammer befindlichen Luftvolumens führt bereits bei einem Druck von 2–4 mm Wassersäule zum Ansprechen des hochempfindlichen Druckwellenschalters.

Ein Schaltbild der Steuereinheit ist in Fig. 2 wiedergegeben. Als Druckwellenschalter (Fig. 2a) für kleinste Drucke wurde der Typ DWK mit eingebauter Verstärkereinheit und Relais der Firma Max Bircher, Schaffhausen, Schweiz verwendet. Durch den Impuls des Druckwellenschalters wird ein Vielbereichszeitrelais (Fig. 2b) (Type 7 PN 20 (RS 120), Siemens AG) in Gang gesetzt, welches über ein Hilfsrelais (Fig. 2c) den Strom zur Pumpe (Fig. 2d) unterbricht. Gleichzeitig wird der Druckwellenschalter stromlos gemacht, um ein Oszielliren seiner Membran bei langsam abfallendem Flüssigkeitsspiegel zu verhindern. Für den Fall einer Störung im freien Ablauf des Effluents aus der Kolonne oder um die Chromatographie durch Schliessen eines Magnetventils (Fig. 2f) im Kolonnenausfluss zu beendigen, wurde parallel zur Pumpe ein Sicherheitszeitrelais (Fig. 2e) (Type DZ 12/SG, Schleicher, Berlin) eingebaut. Es hat die Aufgabe, das unkontrollierte Ansteigen von Flüssigkeit im Kolonnenrohr für den Fall zu verhindern, dass die Membran des Druckwellenschalters in seiner Ruhephase nicht ausreichend entlastet wird und dieser daher nicht in der Lage war, die Pumpe über ein Hilfsrelais (Fig. 2g) abzuschalten. Man kann daher durch das Relais das gesamte Gerät nach einer vorwählbaren und die normale Laufzeit der Pumpe (wenige Minuten) um *ca.* 100% übersteigenden Zeit ausser Betrieb setzen.

Zur Herstellung des Gradienten verwenden wir im allgemeinen eine Gradientenpumpe (Beckman Instruments, München); gleich geeignet ist ein Gradientenmischer vom Typ des Varigrad[2] in Kombination mit einer Schlauchpumpe.

Aus der Funktionsweise der beschriebenen Apparatur folgt zwangsläufig, dass reproduzierbare Trennungen auch auf schrumpfenden Austauschern erhalten werden müssen, wie dies auch an Beispielen gezeigt werden konnte[3]. Das Gerät lässt sich

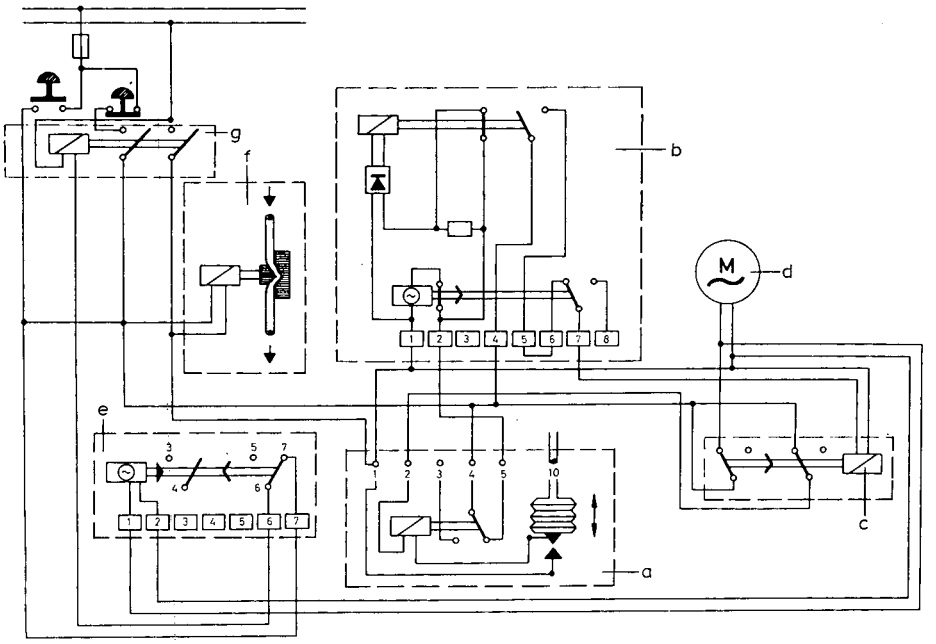

Fig. 2. Steuereinheit. (a) Druckwellenschalter; (b) Vielbereichszeitrelais; (c) Hilfsrelais; (d) Motor der Pumpe; (e) Sicherheitszeitrelais; (f) Magnetventil; (g) Hilfsrelais.

ausserdem in Kombination mit einem automatischen Pufferwechsler zur Durchführung von Chromatographien mit stufenweiser Veränderung des Elutionsmittels verwenden.

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CHROM. 3917

Book Reviews

The Solid–Gas Interface, by E. ALISON FLOOD (Editor), Marcel Dekker, New York, and E. Arnold, London, 1966/1967, two volumes, XXXII and 1175 pp., Price £8.15.0 and £11.0.0.

The book gives an up-to-date, comprehensive review in 37 chapters of the physical properties of the solid–gas interface. Every chapter is presented by an expert in the field and in spite of the large number of contributors (41) the treatment is fairly homogeneous. Practically all physical phenomena occurring at the gas–solid interface are discussed. However the book is mainly devoted to adsorption phenomena. Chapters on catalysis, swelling and liquid coated solids fall strictly speaking outside the scope of the book and are therefore less comprehensive. Nevertheless these chapters are of importance in the field of chromatography. The subjects of special interest for the chromatographer are the following:

Chapter 19: Surface and Volume Flow in Porous Media (R. M. BARRER), 53 pages;

Chapter 20: Chromatography and the Solid–Gas Interface (H. W. HABGOOD), 36 pages;

Chapter 21: Sorption by Liquid-Coated Solids and Gas Chromatography (S. ROSS AND E. D. TOLLES), 20 pages;

Chapter 22: Equilibrium Swelling due to Sorption (W. PRINS), 24 pages.

The layout and the printing of the book are good. The price is quite high but not in relation to the content.

It is hoped that a similar book on the solid–liquid interface will be published very soon.

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CHROM. 3935

Quantitative Paper and Thin-Layer Chromatography, edited by E. J. SHELLARD, Academic Press, London and New York, 1968, 140 pp., price £ 2.2.6.

This book contains the formal contributions made by invited speakers at a recent Symposium, the object of which was to discuss problems associated with quantitative paper and thin-layer chromatography. Unfortunately, the book does not contain the general discussion arising from the formal presentations.

The first four, and the eighth, chapters (J. W. FAIRBAIRN, G. FRANGLEN, W. E. COURT, E. J. SHELLARD and C. A. JOHNSON) discuss the factors involved in producing a spot, the amount of material in which can be accurately and precisely measured, and which can be related back to the concentration in the original sample. Densitometry, visual assessment and elution from the stationary phase followed by spectroscopy are the methods of quantitation described in these chapters. Original data are included to support the authors' conclusions, one of the more important being that a major source of error lies in the difficulty of applying known amounts of material to the stationary phase.

A chapter on the application of spectroscopy to thin-layer chromatography (G. W. GOODMAN) tends to concentrate on the solution to one or two specific problems (albeit in an original manner) by I.R. spectroscopy, rather than give a general account of the difficulties encountered in obtaining meaningful spectra on microgram quantities of components initially adsorbed on active materials. The remaining chapters deal with some of the more specialised (and expensive) instrumentation that is currently being used for the evaluation of chromatograms. These include fluorimetry (D. E. JÄNCHEN), direct spectrophotometry (H. JORK) and three methods of radioactive scanning (B. A. WOOD, A. E. LOWE, and B. R. PULLAN).

As might be expected from a book of this type, there is some variation in the quality of the chapters. Typographical errors are restricted to two or three contributions, whilst the understanding of bidimensional radiochromatogram scanning with Geiger-Müller counters, punched-tape and a computer would have been facilitated by the inclusion of a few simple diagrams.

It is clear that much work needs to be done before the full potential of quantitative paper and thin-layer chromatography is fully realised. In the meantime, those that contemplate using such techniques will benefit from reading this book.

Esso Petroleum Co. Ltd., Abingdon, Berks. (Great Britain) R. AMOS

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See also TLC section.

2. FUNDAMENTALS, THEORY AND GENERAL

See TLC section.

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CHROM. 3936

QUANTITATIVE ANALYSIS ON THIN LAYER CHROMATOGRAMS: APPLICATION OF AN OPTICAL THEORY TO *IN SITU* ANALYSIS

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SUMMARY

A method for the *in situ* quantitative analysis of thin layer chromatographic zones on microscope slides is described. It comprises the quantitative application of 1 μl of solution to the plate, two-dimensional scanning of the zone with a point of monochromatic illumination, then off-line data processing by computer so that a simplified form of the general KUBELKA–MUNK equation for transmission can be applied. The standard deviation observed on independently analysed 0.510 μg loads of acetone dinitrophenylhydrazone on silica gel thin layers using the experimental prototype apparatus is 2.7%.

INTRODUCTION

The purpose of this paper is to demonstrate the feasibility of quantitative analysis *in situ* on thin layer chromatograms by an absorptiometric method based upon the optical properties of light-scattering thin layers, and to indicate the order of precision attainable.

Quantitative analysis on paper and thin layer chromatograms by light absorption has been attempted by various workers[1–17], and several instruments are at present marketed for this purpose. The bulk of the methods published are empirical in nature.

The exact relation between incident and emergent light energy in highly scattering media is complex, so in an earlier paper[18] we have developed an approximate equation relating all the important variables that remain after applying appropriate constraints. The equation is based on a simplified theory of radiative transfer. It is hoped that the advantages of having a theoretical basis, rather than resorting to complete empiricism, will outweigh the disadvantage of increased complexity of the analytical method. The advantages expected are a wide generality of application, and an accuracy which is only dependent on the control or estimation of known variables. The disadvantage of complexity is quite serious, but the increasing use of high-speed computers for data processing has considerably reduced this problem.

THEORY

Summary

In ref. 18 the advantages of transmission rather than reflection for *in situ* quantitative analysis are defined. What is believed to be a sufficiently accurate, but more easily used, approximation to the KUBELKA–MUNK transmission equation[19,20] is derived:

$$0.434\,KX = 2\exp[-2A_0]\,(A + 0.4\,A^2) \qquad (1)$$

where:

$0.434\,KX$ is proportional to the weight of light-absorbing substance per unit area and equals absorptivity $\times$ mg $\times$ cm^{-2}.

A_0 is the background absorbance due only to scattering (no absorbing substance present). A_0 is related to the scattering power (SX) of the thin layer by:

$$A_0 = \log[1 + SX]$$

since

$$T_0 = \frac{1}{1 + SX}$$

and

$$A_0 = \log\left[\frac{1}{T_0}\right].$$

A is the increase over the background absorbance due to the presence of a light-absorbing substance.

The equation is applicable over the range:

$$0.7 < A_0 < 1.3$$
$$0 < A < 1.0$$

Furthermore, the KUBELKA–MUNK equation, and consequently eqn. (1), can only be applied when the measurements of the absorbances are carried out within certain constraints. The more important experimental implications of these are:

(i) The light used must be sufficiently monochromatic.

(ii) The absorption must be adequately uniform over the area illuminated.

(iii) The angular distribution of incident light must be constant.

(iv) The angular orientation of the light-collecting or measuring device must not vary.

Application

From eqn. (1) it can be seen that the concentration (C) of a compound in mg $\times$ cm^{-2} at any point on a thin layer chromatogram is given by:

$$C = \frac{2\exp[-2A_0]}{a}\,(A + 0.4\,A^2)$$

where a is the absorptivity of the adsorbed substance in a chromatographic zone.

If $(\Delta A_0 + A_0')$ is substituted for A_0, where A_0' is a fixed standard absorbance level, so that $\Delta A_0 = A_0 - A_0'$, then

$$C = \frac{2 \exp [-2A_0']}{a} \cdot \exp [-2\Delta A_0] (A + 0.4 A^2) \quad (2)$$

The quantity, M, in μg of a compound in a chromatographed zone is given by:

$$M = 1000 \int_{x_1}^{x_2} \int_{y_1}^{y_2} C \, dy \, dx$$

$$\simeq 1000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} C \, \Delta y \, \Delta x \quad (3)$$

The limits y_1 to y_2 and x_1 to x_2 are taken as distances along perpendicular axes in the plane of the thin layer, sufficient to include all the zone. The variations of C over the distances Δx and Δy have to be small for the summation in eqn. (3) to approach the true value of M. Substituting for C from eqn. (2), into eqn. (3):

$$M = 1000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} \frac{2 \exp [-2A_0']}{a} \cdot \exp [-2\Delta A_0] (A + 0.4 A^2) \Delta y \, \Delta x$$

$$= \frac{\exp [-2A_0']}{a} \cdot 2000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} \exp [-2\Delta A_0] (A + 0.4 A^2) \Delta y \, \Delta x$$

The factor

$$\frac{\exp [-2A_0']}{a}$$

is unknown, but it is constant and can be determined in practice by analysis of a known weight of material. In the remaining factor

$$2000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} \exp [-2\Delta A_0] (A + 0.4 A^2) \Delta y \, \Delta x \quad (4)$$

the values of ΔA_0 and A are to be determined at intervals of Δx by Δy over the whole area from y_1 to y_2 and from x_1 to x_2. Before this is carried out, the following points require consideration:

(1) The light used must be sufficiently monochromatic. This would follow by inference from a demonstration that with the selected source and interference filter, solutions of the test compound obey Beer's law over the absorbance range in question.

(2) The measurement of absorbance is usually made over a small element of area which has, to a first approximation, a uniform gradient of absorbance. Under these conditions the experimental result (−log of the mean transmittance) is less than the actual value.

It has been shown[18] that when using uniform illumination, this bias is dependent only upon the range of absorbance covered by the illuminating aperture. It will be assumed, as a simplification, that the absorbance gradient is constant over the region of the spot, *i.e.* a conical absorbance profile, and negligible elsewhere. Then the approximate percentage reduction, R, as a result of a bias, θ, on a measurement of the "volume" of the absorbance profile of a spot of peak absorbance, P and diameter, D, is given by:

$$R = 100 \cdot \frac{\theta \dfrac{\pi D^2}{4}}{\frac{1}{3} P \dfrac{\pi D^2}{4}} = \frac{3\theta}{P} \cdot 100\%.$$

Thus 1% reduction in spot "volume" is brought about by a bias equal to $\frac{1}{3}$% of the peak height, *i.e.* for a peak height of 0.5, this is 0.0017 absorbance.

The relation between bias, θ, and absorbance range uniformly illuminated, d

$$\theta = \log \left[\frac{\sinh (1.151\, d)}{1.151\, d} \right]$$

is derived in ref. 18, where the graph is also given. The exact derivation of bias for a circular aperture and conical zone absorbance profile is complicated, and is not warranted in this context. So, using the graph of the above relation between θ and d, when $\theta = 0.0017$, $d \doteq 0.1$. With a spot of 0.5 absorbance peak height, the ratio of the diameters of the illuminating aperture and spot must be less than 1:10 to produce an illuminated absorbance range of less than 0.1, and thus less than 1% bias on the final summated spot profile.

In the experiment below, the maximum peak absorbance was no greater than 0.5. As the spot diameters were in the range 5–8 mm the aperture of 0.5 mm, together with some lateral diffusion of light in the thin layer, should only produce approximately 1% bias in the calculated results.

(3) The absorbance values, when estimated, are the combination of background and sample absorbance, $\Delta A_0 + A$. The procedure used to separate A and ΔA_0 was to interpolate the values of ΔA_0 within the zone from measurements around the periphery of the scanned region, where the value of A is known to be zero. Subtracting the interpolated ΔA_0 values from the measurements within the zone then gives A at each point.

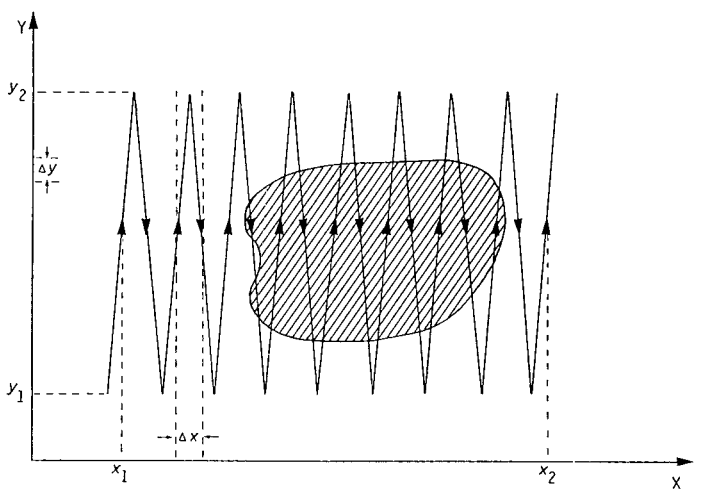

Fig. 1. Diagrammatic representation of the path of the light spot (→—) over a chromatographic zone (hatched area).

(4) Finally some means of obtaining the many absorption estimates at known spacings, Δx and Δy, in the two dimensions, x and y, is required. This can be accomplished by the use of some type of scanning motion with successively displaced scans. Two alternatives are possible. To scan the thin layer by a moving light spot, or to move the thin layer and keep the light beam stationary. We devised a mechanism to move the chromatographic slide rather than the light spot. In this way we were able to keep both the angular distribution of incident light, and the collection angle of the photomultiplier constant, as required by conditions (iii) and (iv) above.

The mechanism used for scanning (see later) gave a saw-toothed motion to the plate (Fig. 1). Δx was taken as the average spacing between scans. The actual spacing with this saw-tooth motion varies from 0 to $2\,\Delta x$ from one end of the scan to the other. Although this does not affect the calculations, the efficiency of this type of scanning is decreased towards the ends of the scans because of the unequal spacing. However the absorbance gradients in these regions are lower than in the central portion of the scan so that the lower efficiency can be tolerated. The transverse spacing Δy is obtained as the distance, perpendicular to the overall motion, between successive readings.

Having obtained ΔA_0, A, Δx and Δy, these values are substituted into the expression (4) and summated to give a single value which is proportional to μg of compound in the zone. The constant of proportionality is

$$\frac{\exp\,[-2A_0']}{a}.$$

As the expression $\exp\,[-2\,\Delta A_0]\,(A + 0.4\,A^2)$ has to be evaluated $[(x_2 - x_1)(y_2 - y_1)]/(\Delta x\,\Delta y)$ times where Δx and Δy are of the order of 0.05 cm, and $(x_2 - x_1)$ and $(y_2 - y_1)$ are of the order of 1.5 cm, the number of times the expression is calculated is about 900 per analysis, before summation. Thus, to be useful, this method of analysis must have some rapid automatic means of computation.

EXPERIMENTAL

Instrumentation

The instrument used to apply the above concepts to quantitative analysis of thin layer chromatograms was constructed by modifications and additions to a Chromoscan densitometer (Joyce Loebl & Co. Ltd., Gateshead, England). Ideally, a new class of instrument would be preferable and more generally useful. However, for use in an exploratory experiment, of the type described below, the limitations of slow speed and poor wavelength selection imposed by adapting an existing instrument are not very serious.

The recording densitometer was fitted with a quartz iodine source, a 0.5 mm circular aperture, and a 420 nm interference filter with a half band width of 15 nm. The linear motion carriage and associated mechanism was dismantled and removed. The transmission photomultiplier stand was replaced by one occupying less space.

Inside the now empty sample compartment four legs were fitted, each with a 1 mm pitch (OB.A.) half nut soldered to the top. These support the scanning mechanism, shown in Fig. 2, by the two horizontal 1 mm pitch screwed rods. When these screwed rods rotate they impart a horizontal motion (x axis) to the mechanism, and

TABLE I

K AUTOCODE COMPUTER PROGRAM USED IN THE CALCULATION OF THE RESULTS

```
title
ici/k/17237/8  j.goldman(pharm) adtlc analysis

chapter0
a=1≈,b=3200≈,d=30≈,≈b=3200≈,≈c=110≈,≈d=110≈,k=110
c=110
1)newpage
read data title
read(f,a0,a1)≈,(scale and base line)
≈t=0
t=s=0
e=0
2)readword(a,p,q,r,3)
3)jump(2)a>9≈,(search for 1st scan)

(read in data)
j=0,2,100
i=1
b(30j+i)=a
4)i=i+1
readword(b(30j+i),p,q,r,5)
5)jump(7)b(30j+i)=-1≈,(end of data)
s=s+1
jump(17)q≠4≈,(mispunch)
jump(17)b(30j+i)>999
6)jump(22)i>60≈,(scan too wide)
jump(4)b(30j+i)>0
jump(4)i=3≈,(extra scan markers)
a=b(30j+i)
i=i-1
k(j)=0intpt(0.5i)≈,(k(j)=width of jth scan)
k(j+1)=i-k(j)
i=1,1,k(j+1)≈,(split double scans)
di=b(30j+k(j)+k(j+1)+1-i)
repeat
i=1,1,k(j+1)
b(30≈(j+1≈)+i)=di
repeat
≈s=s≈,(s=no of data points)
repeat≈,j=j+2
7)n=j+1
k=k(0min(k0,0,n-2))
?g=≈s/j≈,(g=mean no of data per scan)

(interpolate mispunched data values)
j=1,1,n-1≈,(remove scan markers and adjacent points)
i=2,1,k-1≈,(-leave space round matrix)
≈b(jk+i)=b(30j+i-29)
repeat
repeat
jump(12)t=0≈,(no errors)
i=1,1,k
≈b(nk+i)=≈bi=10000
repeat
i=ak,k,nk
≈bi=≈b(i-k+1)=10000
repeat
j=1,1,n-1≈,(search for errors)
8)i=0max(≈bo,jk+2,k≈(j+1≈)-1)
jump(11)≈bi≈>1000≈,l=0
e=0
≈q=-1,1,1
m=-1,1,1≈,(search for adjacent correct data points)
jump(9)≈b(i+≈qk+m)>1000
e=e+≈b(i+≈qk+m)
l=l+1
9)repeat
repeat
jump(10)l>0
nlcaption
too many mispurches around scan
print(j-1,2,0)
caption
point
print(i+1-k0intpt(i/k),2,0)
jump(1)
10)≈bi=e/l≈,(corrected data value inserted)
t=t-1≈,(one less error left)
jump(12)t=0
jump(8)

(determine length of zone)
11)repeat
12)n=n-2≈,(n=no of last scan)
k=k-2≈,(k=no of points per scan)
j=0,1,n≈,(remove margin,correct-)
i=1,1,k≈,(-for drift,convert to absorbance)
b(jk+i)=f≈(≈b(≈(k+2≈)≈(j+1≈)+i+1)+≈(a0≈(j-n≈)-ja1
repeat
repeat
j=0,1,n
cj=b(0max(bo,jk+1,jk+k))≈,(c=(db/dj)i=l)
repeat
j=1,1,n
≈cj=cj-c(j-1)
repeat
≈i=0max(≈c0,1,n)≈,(front of spot)
≈j=0min(≈c0,1,n)≈,(back of spot)
j=≈i
13)j=j-1
jump(14)≈cj≈>0.01
jump(13)j>5
14)≈i=j-3≈,(i=start of spot)
j=≈j
15)j=j+1
jump(16)≈cj>-0.01
jump(15)j≈>n-4
16)≈j=j+1≈,(j=end of spot)

(interpolate background to find sample absorbance
j=≈i,1,≈j
≈c=≈b=0
m=j-2,1,j+2
jump(24)n≈>0
jump(25)m>n
≈b=≈b+b(mk+1)+b(mk+2)
≈c=≈c+b(mk+k)+b(mk+k-1)
jump(23)
24)≈b=≈b+b1+b2
≈c=≈c+bk+b(k-1)
jump(23)
25)≈b=≈b+b(nk+1)+b(nk+2)
≈c=≈c+b(nk+k)+b(nk+k-1)
23)repeat
```

thus to the chromatographic slide. The vertical motion (y direction) is provided by having the slide holder aligned by a tongue and groove at one end and fixed to a 1 mm pitch nut on a vertical screwed rod at the other. This vertical rod is driven via a 27:1 gear ratio from a half-crown and pinion assembly which provides alternately 13.5 cycles in one direction and 13.5 cycles in the other. Both the half-crown and the horizontal shafts are driven from the same 1.2 r.p.m. synchronous motor. The horizontal screw drive rotates 0.583 times during each half cycle of the half-crown producing 0.583 mm horizontal motion between scans. The scanning mechanism is made from F.A.C. Construction System (sold by Transitoria Trading Company A.B., Stockholm, Sweden) except for the screwed rods and microscope slide holder.

```
i=1,1,k
*b(jk+i)=b(jk+i)-0.1*(*b*(k-i*)+*c*(i-1*)*)/*(k-1*)
repeat
repeat
i=3,1,k-2
*c=*b=0
m=i-2,1,i+2
*b=*b+*b(*ik+m)+*b(*ik+k+m)
*c=*c+*b(*jk+m)+*b(*jk-k+m)
repeat
j=*i+2,1,*j-2
*b(jk+i)=*b(jk+i)-0.1*(*b*(*j-j*)+*c*(j-*i*)*)/*(*j-*i*)
repeat
repeat

(apply simple k-m eqn and summate)
*e=*d=0
j=*i+2,1,*j-2
*dj=0
i=3,1,k-2
b(jk+i)=b(jk+i)-*b(jk+i)*,(background)
*dj=*dj+*b(jk+i)*(1+0.4*b(jk+i)*)@exp(-2b(jk+i))*,(k-m)
*e=*e+b(jk+i)
repeat
?*d=*d+*dj*,(integrate)
repeat

(print out results)
*e=*e/*(k-4*)*(*j-*i-3*)*,(e=avg background)
nlcaption
micrograms x absorptivity x exp(2ao)=
print(157.5*d/g,4,2)
nlcaption
mean background absorbance=
print(*e,1,3)
nlcaption
with max of
print(b(@max(bo,*ik+1,k=(*j+1*))),1,3)
caption
and min of
print(b(@min(bo,*ik+1,k=(*j+1*))),1,3)
nlcaption
length of scan used=
print(0.5833*(*j-*i+1*),2,1)
caption
mm
nlcaption
with
print(0.5833*i,2,1)
caption
mm spare at start
nlcaption
and
print(0.5833*(n-*j*),2,1)
caption
mm spare at end
nlcaption
width of scan=
print(13.5k/g,2,1)
caption
mm
nlcaption
number of interpolated mispunches=
print(*t,3,0)
newline

(print out zone absorbance matrix)
i=k,-1,k-1
newline
j=*i,1,*j
print(1oob(kj+i),2,0)
repeat
repeat
i=k-2,-1,3
newline
print(1oob(k*i+i),2,0*,1oob(k*i+k+i),2,0)
j=*i+2,1,*j-2
print(1oo*b(kj+i),2,0)
repeat
print(1oob(k*j-k+i),2,0*,1oob(k*j+i),2,0)
repeat
i=2,-1,1
newline
j=*i,1,*j
print(1oob(jk+i),2,0)
repeat
repeat
jump(1)

(correct or replace mispunches)
17)jump(18)p+r*0
jump(18)q*3*,(and digit 6 missing)
b(3oj+i)=6o+b(3oj+i)+9o@intpt(0.1b(3oj+i))
jump(6)
18)jump(2o)j=0
jump(21)i=>k(j-1)+k(j-2)*,(not end of scan)
19)b(3oj+i)=0
jump(6)
2o)jump(19)i>53*,(end of 1st scan)
21)b(3oj+i)=1oooo
t=t+1*,(t=no of interpolable mispunches)
*t=t
jump(6)
22)nlcaption
scan marker missing after scan
print(j,3,0)
jump(1)
close
```

The pen drive wire on the Chromoscan was extended by the manufacturer to operate a "slave" potentiometer. Approximately 10 V was applied across the two ends from 5 lead/acid accumulators via two trimming potentiometers for "scale" and "baseline" adjustment. The output from the rotating contact, adjusted to be within the range 1.00 to 9.99 V, was fed into a prototype digitiser and encoder (see Acknowledgements) to supply a digital output of 1 unit per 0.01 V in Mercury code at constant intervals of 0.926 sec. This output was punched on to 5 hole tape by a Creed 25 character/sec punch.

Connected to the motor shaft of the scanning mechanism was an arm which closed a microswitch momentarily once each cycle. This operated a short circuit in

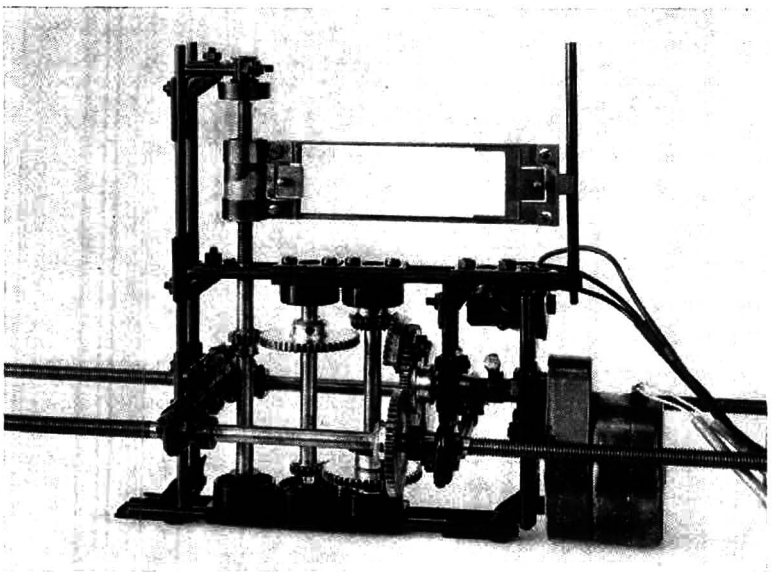

Fig. 2. Scanning mechanism, assembled from F.A.C. Construction System, so as to fit into the sample compartment of a Joyce Loebl Chromoscan. It imparts a saw-tooth motion to the microscope slide chromatogram placed in the holder.

the output voltage for about 1 sec to give one or two readings of 0.00 V. The arm was adjusted to operate when the light spot was at one extremity of the traverse across the chromatographic plate.

Program

A computer program was written in K Autocode (see Table I) to carry out the calculations on the data required for the analysis. The program aligns the data into the separate consecutive scans across the thin layer using the zero values as markers. The data which is in the form of integers between 100 and 999 is then converted into absorbance relative to the standard absorbance level and corrected for any instrumental drift during the experiment. The position and extent of the chromatographic zone is determined and the absorbance values around it are used to interpolate ΔA_0 values for each point in the region containing the zone. These are then subtracted from the absorbance values in this region to give sample absorbances A for each point. The value of the expression $\exp[-2\Delta A_0](A + 0.4A^2)$ is calculated for each point and then summated over the whole region containing the zone. The result is multiplied by $2000\Delta x\Delta y$ after calculating the value of Δy (the size of Δy varies slightly as it is dependent on the time interval between successive readings).

The result, which is equal to absorptivity $\times \exp[2A_0'] \times \mu$g of compound in the zone, is printed out, together with other data of interest, such as the mean value of ΔA_0 within the zone, the size and position of the zone etc. (see Table II). Also printed is a grid of the sample absorbance $\times$ 100, surrounded by the relative background absorbance $\times$ 100 used in the interpolation procedure.

The program also includes a correction for a specific fault which prevented the

number 6 in the tens position from being punched. There is also a general interpolation for any other type of mispunch. The data was computed on a KDF9 computer, each data tape requiring about 30 sec of computer time.

Procedure

Microscope slides, 7.5 cm × 2.5 cm, were cleaned in chromic acid, rinsed with water then methanol and dried at 100°. This seemed to reduce the probability of the gypsum binder in the adsorbent crystallising on to the glass, which would have caused optical inhomogeneities in the thin layers. Thin layers of silica gel (Merck Kieselgel G) were prepared on the microscope slides using a brass bedplate and slurry box with about 250 μ clearance above the glass surfaces. Five grams of adsorbent slurried with 10 ml of distilled water were used to prepare each batch of 12 thin layers. After drying at 90° the adsorbent was cleaned from a 1.5 cm portion at one end of each slide.

An acetone DNPH solution was prepared in methylated spirits at 0.510 mg/ml. Portions were diluted to 75, 50 and 25% of this strength. The solutions were kept in small airtight bottles with rubber lined screw caps.

One microlitre of the solution to be analysed was transferred as follows to a thin layer plate using a 1 μl capillary micropipette (Microcaps, Drummond Scientific Co., U.S.A.). The capillary was always filled from one end, and emptied from the other. It was rinsed once with clean solvent and once with the sample solution before filling. The micropipette was discharged on to the thin layer 1 cm from the bottom of the plate. Care was taken not to dislodge any adsorbent particles. It was then filled with clean solvent and again emptied on to the thin layer in the same location. The choice of the polar solvent, methylated spirits for rinsing, is to elute the spot into a ring, so that no sample is lost on adsorbent adhering to the pipette. The capillary was then cleaned by rinsing once more with clean solvent. The same pipette, which had been found to contain 1.00 μl by weighing, was used throughout.

The methylated spirits was evaporated from the spot by blowing gently for about 30 sec before development through 5 cm with toluene in a 3 oz. (approx. 80 ml) screw-topped bottle with a wide neck. The R_F value of acetone DNPH in this system is approximately 0.4. The chromatographic plate was then placed in a vacuum jar and most of the toluene evaporated under reduced pressure. However, some solvent remained due to the low temperature attained by the slide, so it was removed and warmed on a hot plate for 10 sec before returning to the vacuum jar for a further 30 sec at 0.1 mm Hg pressure.

The back of the microscope slide and the end portion with no adsorbent were carefully cleaned before placing the plate into the holder on the scanning mechanism. With the light spot falling on a background portion of the plate *i.e.* not on the spot, the pen position was adjusted to be near the lower absorbance limit on the scale. The scanning mechanism was then moved to a position where the light spot passed through a piece of diffusing glass fastened to one end of the slide holder, and the output noted. The attenuation caused by this diffuser acts as the standard absorbance level. The mechanism was then moved back to a position such that the light spot was approximately 8 mm to one side of the chromatographed zone. The chromatogram was then scanned, the output being recorded on 5 hole tape. After scanning, the standard absorbance level was again noted so as to allow for any instrument drift

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TABLE II

COMPUTER RESULT CALCULATED FROM THE PRINTED DISTRIBUTION OF ABSORBANCE (× 100) OF A CHROMATOGRAPHIC ZONE

```
ICI/K/15586/13.26  100

MICROGRAMS X ABSORPTIVITY X EXP(2A0)=  346.52
MEAN BACKGROUND ABSORBANCE=-0.553
WITH MAX OF-0.523  AND MIN OF-0.573
LENGTH OF SCAN USED= 13.4  MM
WITH  0.0  MM SPARE AT START
AND  0.6  MM SPARE AT END
WIDTH OF SCAN= 12.1  MM
NUMBER OF INTERPOLATED MISPUNCHES=   1.

-54  -55  -55  -55  -55  -55  -55  -55  -56  -56  -56  -56  -56  -56  -57  -57  -56  -56  -57  -57  -57  -57  -57
-54  -54  -55  -55  -55  -55  -55  -55  -56  -56  -56  -56  -56  -56  -56  -57  -56  -56  -57  -57  -57  -57  -57
-54  -55   -0   -0   -0    0    0   -0   -0   -0    0    0    0    0    0   -0    0    0    0   -0   -0  -57  -57
-55  -55    0   -0   -0   -0    0   -0    0   -0    1    0    0    0    0   -0    0    0    0   -0   -0  -57  -57
-55  -55    0   -0    0    0    0   -0    1   -0    5    2    2    0    0   -0    0    0   -0   -0    0  -57  -57
-55  -55   -0   -0    0    0    1    0    3    2   10    8    5    6    1    0    1   -0    0   -0    0  -57  -57
-54  -55   -0   -0   -0   -0    2    1    6    7   20   18   14   12    3    1    1    0    0   -0    0  -57  -57
-54  -55    0    0   -0   -0    2    2   12   15   28   28   22   17    7    3    1    0    0   -0    0  -57  -56
-54  -55    0    0    0   -0    2    3   13   17   30   32   27   19    9    4    2    0    1   -0    0  -57  -56
-54  -54   -0    0    0    0    2    5   14   20   29   32   24   16    8    4    1    0    0   -0    0  -57  -56
-54  -55    0   -0   -0    0    2    6   14   21   28   29   22   15    7    4    1    0    0   -1    0  -56  -56
-54  -54   -0   -0    0    0    3    6   13   22   29   27   21   14    7    3    1    0    0   -1    0  -56  -56
-54  -54    0   -0    1    1    2    6   13   23   29   27   23   14    8    3    1    0    0   -1    0  -56  -56
-54  -54    0   -0    0    1    1    6   12   23   29   29   26   16    9    3    2   -0    0   -1    0  -56  -56
-54  -54    0   -0    1    1    1    6   10   23   27   31   28   18   11    4    2   -0    0    0    0  -56  -55
-54  -54    0   -0    0    0    1    4    8   22   22   29   25   16   11    4    1   -0    0   -0   -0  -56  -55
-53  -54    0   -0    0   -0    0    2    4   16   12   18   18    9    9    3    1   -0   -0   -0   -0  -56  -55
-53  -54    0   -0   -0   -0    0    1    2    8    4    8    9    4    4    2    0   -0    0   -0   -0  -56  -55
-53  -53    0   -0   -0   -1    0    1    0    3    1    3    2    1    1    1    0   -0    0   -0    0  -56  -55
-53  -54   -0   -0   -0   -0    0    0    0    1    0    1    0    1    0    1    0   -0    0    0    0  -56  -55
-53  -54   -0   -0   -0   -0    0   -0    0    1    0    0   -0    0   -0    0    0   -0    0    0    0  -56  -55
-53  -53    1   -0    0   -1    0    0   -0    0    0    0   -0   -0   -0    0    0   -0   -0    0   -0  -55  -55
-52  -53  -52  -54  -53  -54  -54  -54  -54  -55  -54  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55
-52  -52  -53  -54  -53  -54  -54  -54  -54  -54  -54  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55  -55
```

during the experiment. The scale was then estimated so that the output could be converted to absorbance.

The analysis of each chromatogram was completed within half an hour of development as acetone DNPH spots were not entirely stable on silica gel. A loss of about 4% after 6 h had previously been observed.

A data heading, the output scale and the first and second standard absorbance readings were punched on 5 hole tape and spliced on to the start of the data tape. The number, −1, to signify the end of data to the program, and the end of tape symbols were hand-punched on to the end of the tape. The data was computed in a manner dictated by the program, so as to provide a result which should be proportional to the weight of acetone DNPH put on to the chromatogram.

Results

The above procedure was carried out sixteen times, each on separate plates with no special layer thickness control. There were ten sample loads of 0.510 μg and two loads at 0.383, 0.255 and 0.128 μg, respectively.

TABLE III

EXPERIMENTAL RESULTS FROM ANALYSES OF KNOWN QUANTITIES OF ACETONE DINITROPHENYLHYDRAZONE

Acetone DNPH (μg)	*Result*[a]	*Results × conversion factor*[b] *(μg)*	*Error (μg)*	*Mean ΔA_0*
0.510	332.6	0.535	0.025	−0.482
0.510	328.3	0.528	0.018	−0.505
0.510	317.9	0.512	0.002	−0.581
0.510	317.5	0.511	0.001	−0.487
0.510	306.3	0.493	−0.017	−0.494
0.510	305.1	0.491	−0.019	−0.482
0.510	316.5	0.510	0.000	−0.553
0.510	318.6	0.513	0.003	−0.547
0.510	315.6	0.508	−0.002	−0.525
0.510	309.7	0.499	−0.011	−0.508
0.383	263.6	0.424	0.041	−0.550
0.383	242.5	0.390	0.007	−0.500
0.255	171.1	0.275	0.020	−0.483
0.255	165.4	0.266	0.011	−0.490
0.128	86.2	0.139	0.011	−0.501
0.128	85.9	0.138	0.010	−0.499

[a] Computed as μg × absorptivity × exp[2 A_0'].
[b] See text.

Table III shows the computed results against μg of acetone DNPH chromatographed. Dividing the mean of the ten results at 0.510 μg into 0.510 gave the conversion factor, 0.001610 μg/unit. This value was used to convert all the results to μg for comparison with the known quantities loaded on the thin layer plates. The differences between the estimated and loaded weights are given under "error". These figures were used to calculate the overall standard deviation as 0.017 μg and on the

0.510 μg level alone, 0.014 μg or 2.7%. The mean ΔA_0 is the difference between the mean background absorbance of the thin layer and the standard absorbance level, A_0'. Equating the conversion factor to

$$\frac{\exp[-2A_0']}{a}$$

where a is the absorptivity of acetone DNPH, and rearranging gives

$$A_0' = -\tfrac{1}{2} \ln [0.00161\ a].$$

Taking the value of a as that in methanol solution, 22.4 (which will only be approximately that of acetone DNPH adsorbed on silica gel), gives A_0' as approximately 1.661. The actual background absorbance of the thin layers, A_0, may now be calculated from:

$$A_0 = A_0' + \Delta A_0$$

From the values of ΔA_0 in Table III the range of A_0 in this experiment is seen to be 1.080 to 1.179, which correspond to a range of scattering powers of 11.0 to 14.1 (using $A_0 = \log [1 + SX]$).

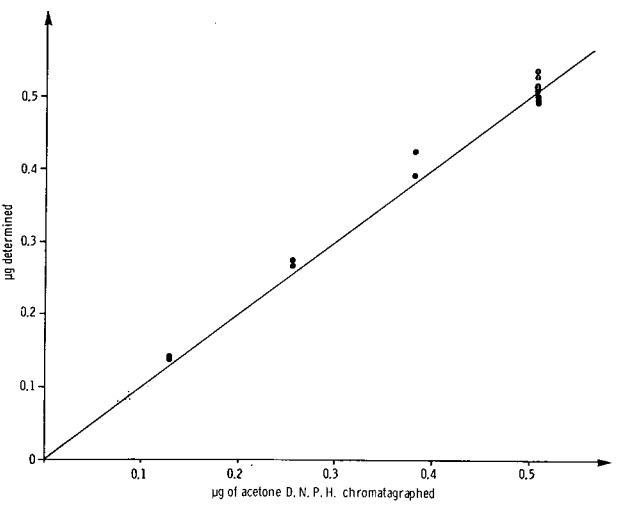

Fig. 3. Experimentally determined *vs.* actual weights of acetone DNPH in chromatographic analyses.

The graph of estimated *vs.* loaded amounts of sample, Fig. 3, may indicate a slight curvature in the experimental points as compared to the ideal straight line. This was probably caused by concentric rings of light of diminishing intensity surrounding the main point of illumination. These were due to internal reflection of diffused light back on to the chromatogram by the microscope slide surfaces. Unfortunately this phenomenon and the reason for it was not realised until after the apparatus had been dismantled. When the chromatogram is illuminated through the glass slide, as in this experiment, the diffuse light reflected back onto the thin layer

was initially reflected by the thin layer. As reflectance varies much less than transmittance these rings of light will be relatively constant in intensity. They were found by experiment to constitute about 5% of the total light energy. Improved linearity would be expected if this additional illumination were obviated. For instance, if the light were focussed onto the chromatogram from the thin layer side, then the internal reflection would be of light initially transmitted by the thin layer at the point of illumination only. Alternatively, the transmitted light could be refocussed through a small aperture before measurement.

DISCUSSION AND CONCLUSION

A theory for radiative transfer in scattering and absorbing sheets derived by KUBELKA and MUNK has been used extensively in measurements concerning the properties of paper and paints. In these fields it has become accepted as being sufficiently accurate for most purposes, although it is not an exact expression. We have reported[18] previously on the derivation and verification of the simplified form (eqn. 1) used in this paper. The advantage of this simplified equation is that the unknown, KX, which is proportional to the weight of light absorber per unit area, is explicitly related to the measured variables, A_0 and A, rather than implicitly as in the general KUBELKA–MUNK equation. This made it feasible to carry out quantification of chromatographed bands in simple cases within the described limitations, without having to use computational aids. That sufficed to obtain a reasonable verification of the simplified expression under conditions that were of limited usefulness.

The purpose of the present paper is to extend quantitative thin layer chromatography to the more general case of spots. As the absorbance of spots varies in both length and breadth, scanning in these two dimensions is necessary and inevitably large numbers of data points are accumulated. Data processing by computer is indispensable for completion of the analysis. The use of a computer brings other advantages such as corrections for instrumental faults, curve fitting and smoothing etc., which otherwise would be neglected.

The results obtained on repeat determinations of 0.510 μg of acetone DNPH on separate thin layer chromatograms showed a standard deviation of 2.7%. Improvement in the precision can be expected from developments in the instrumental design aimed at lowering mechanical and electronic tolerances. No particular control of layer thickness was attempted as there is compensation for this in the calculation of the results. Once the constant of proportionality (which is related to the absorptivity of the compound in question) is known, each analysis is an independent determination so that no calibration against adjacent standards is necessary.

The novelty of this form of analysis is not in the use of either: monochromatic light, two-dimensional scanning, a reasonably correct theory, "off-line" data handling by computer, or the accurate application of small volumes of liquid as spots. Rather it is the combination of all these aspects which provides a substantially non-empirical approach to the problem of *in situ* quantitative analysis.

To increase further the generality of application of quantitative analysis it would be necessary to use light in the ultra-violet region so that direct analysis of many more substances could be made. However, silica gel (as an example of a com-

monly used adsorbent), absorbs light itself in the region 200–275 nm, which includes the region of absorption by many of the common chromophores. Thus to work in this region on silica gel adsorbent introduces two more difficulties: a third independent variable *i.e.* the absorptivity of the substrate, and the low transmittance of the layer itself.

The first case necessitates the use of the general form of the KUBELKA–MUNK transmission equation, as the range of application of our simplified version will not be sufficient to encompass the high overall KX term, obtained by adding that of the substrate to the sample. This difficulty may be ameliorated by computational methods, although this would inevitably incur increased computing time. The KX value of the substrate in the 200–275 nm region could be determined readily by using the simple expression, derived by KUBELKA[20], for K/S in terms of the reflectance of an "infinitely thick" layer.

The magnitude of the second difficulty, that of lower light energy, can only be assessed by further experimental work.

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CHROM. 3883

METHODS OF ANALYSIS OF SULPHUR COMPOUNDS IN HYDROCARBON MIXTURES

III. STUDY OF LIQUID PHASE HYDROGENOLYSIS OF THIONAPHTHENE OVER RANEY NICKEL AT ROOM TEMPERATURE; DETERMINATION OF THIONAPHTHENE ADMIXTURES IN TECHNICAL GRADE NAPHTHALENE BY GAS CHROMATOGRAPHY

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SUMMARY

The hydrogenolysis of thionaphthene by Raney nickel in the medium of benzene at room temperature leads to ethylbenzene, which is the only quantitatively significant reaction product. The degree of conversion of 0.01–2.0% thionaphthene solutions in benzene amounts to approximately 80%. However, both the qualitative and quantitative course of the desulphurization is reproducible even in the presence of a large excess of naphthalene. The reaction can be utilized for the routine determination of 0.05–2.0% thionaphthene contents in technical-grade naphthalenes, the relative error of the analysis being not higher than ± 5%.

INTRODUCTION

The by now classical desulphurization of sulphur compounds with Raney nickel by MOZINGO *et al.*[1] has been much used in organic syntheses and, particularly, in studying the structure of various types of organic sulphur compounds[2].

The desulphurization proper by Raney nickel is not a catalytic reaction; organic sulphur is fixed to the nickel and forms an acid-soluble modification of NiS (ref. 3). Raney nickel contains fixed hydrogen, so that the elimination of the sulphur is accompanied by hydrogenation of the desulphurized hydrocarbon remainder, if the latter is unsaturated and amenable to hydrogenation under the conditions of the reaction.

If the desulphurization of the organic sulphur compound proceeds perfectly according to the scheme quoted in the initial paper by MOZINGO *et al.*[1] (the author mentioned did not notice the combination of radicals produced transitorily),

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$$R\text{–}S\text{–}R' + Ni(H) \rightarrow \underbrace{NiS}_{A} + \underbrace{R\text{–}H + R'\text{–}H,}_{B}$$

and if the experimental conditions are chosen such that quantitative conversion of organically bound sulphur to NiS is guaranteed, then the above reaction can be made use of in two ways:

(a) determination of the amount of NiS

(b) determination of the amount of the hydrocarbon (R = R'), or of a mixture of hydrocarbons (R ≠ R').

Deviations from the presupposed ideal scheme may occur in the course of desulphurization, but, if the reaction is reproducible under a given set of experimental conditions, it is possible to carry out a direct calibration of the tare of the organic sulphur compound *vs.* the amount of the reaction product A or B.

A method based on the determination of the product A, *i.e.* on the determination of the amount of NiS produced, has been developed by TRIFONOV *et al.*[4] and by GRANATELLI[5]. TRIFONOV and co-workers determined thiophenic, sulphidic, disulphidic, and elementary sulphur up to total sulphur contents of 2%. The sulphur content in NiS was determined polarographically with the use of calibration curves for the determination of the individual compounds. According to GRANATELLI[5], it is possible to determine both elemental and organically bound sulphur in concentrations within 10^{-4}–10^{-2}% with a relative accuracy of ±1.6%. The amount of NiS is determined as H_2S by titrating it with a mercury acetate solution, whose titre has been determined empirically on elementary sulphur, as the degree of conversion to NiS is approximately 95%. With the use of the empirical sulphur titre, the apparent recovery was 99%.

Up till now, the identification of the desulphurization product B has only been used for the qualitative analysis of the initial sulphur compound[1,6–8]. Quantitative investigation of the desulphurization product B was launched recently by STASZEWSKI *et al.*[9]. They performed the hydrogenolytic reaction at ambient temperature in the medium of an aprotic solvent (isooctane, benzene), and ascertained that the majority of the hydrocarbon was formed in 3–5 min, the degree of conversion being about 90%. It was still questionable, however, whether the desulphurization would proceed in a similar way with more complicated molecules as well.

In the present paper, we have investigated the feasibility of determining quantitatively condensed sulphur-containing heterocyclics by desulphurizing them and subsequently determining the hydrocarbon produced. The model substances used were thionaphthene and mixtures of thionaphthene and naphthalene in the ratios usually found in technical grade naphthalenes produced by the coal-tar and petrochemical industries. Thionaphthene represents an important impurity in the above products; the aim of the present work was to develop an analytical method for its routine determination.

In this respect, the findings by NIGAM *et al.*[10] on the utilization of gas phase hydrogenolysis of thionaphthene by Raney nickel for the determination of minute contents of thionaphthene in technical grade naphthalenes were complemented, using the experimental arrangement appropriate to reaction gas chromatography.

EXPERIMENTAL

Materials and instrumentation

The purity of the chemicals used (analytically pure benzene, chromatographically pure *n*-octane, pure ethylbenzene) and of the standard compounds (pure thionaphthene, Gesellschaft für Teerverwertung mbH, Duisburg, Germany; zone refined naphthalene, Lachema, N.E., Czechoslovakia) was checked by gas chromatography employing packed columns with two stationary phases of different polarity (Apiezon L and polyethyleneglycol adipate), and a 50 m long squalane coated capillary column with 0.25 mm I.D. The thionaphthene was subjected to elementary analysis; the zone refined naphthalene was found to contain 0.0016% of sulphur[5].

The chromatographic analyses were performed on a Chrom 2 apparatus (Laboratory Equipment, N.E., Prague, Czechoslovakia) fitted with a flame ionization detector. The reaction mixtures, produced from more concentrated model solutions of thionaphthene in benzene (1.0–2.0%), were analysed on a stainless steel column (length 85 cm, I.D. 0.6 cm) packed with 10% Apiezon L-on-Chromosorb W 80/100 mesh packing at a temperature of 90°. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The reaction mixtures coming from the other model solutions were analysed on a column of the same dimensions, packed with Celite, 60/80 mesh, coated with 20% of squalane, the column temperature and carrier gas flow rate being kept at 70° and 50 ml/min, respectively.

Solutions of model substances and samples of technical grade naphthalene

Benzene was chosen as both a solvent and reaction medium as more than 25 wt.% of naphthalene[11] could be dissolved in it even at ambient temperature. Model solutions of thionaphthene in benzene of approximate thionaphthene concentrations of 0.05–2.0% (Table Ia) were prepared by weighing. Model solutions in benzene were also prepared of thionaphthene-zone melted naphthalene mixtures, approximate thionaphthene concentrations in naphthalene 0.05–1.9% (Table IIa), the corresponding thionaphthene concentrations in the benzene varying between 0.01 and 0.5%.

The samples of the technical naphthalenes (A = distilled naphthalene; B = sublimed naphthalene; C = hot-pressed naphthalene), together with pure naphthalene (D = analytical grade naphthalene, Lachema, N.E., Czechoslovakia), were analysed in the form of approximately 25% solutions in benzene. All the solutions contained *n*-octane, which served as an internal standard for the quantitative determination of the ethylbenzene produced as a result of the desulphurization of the thionaphthene.

Procedure

An appropriate amount of Raney nickel suspension[9] in isopropyl alcohol is transferred into a ground-stoppered vessel, the isopropyl alcohol is poured off, the solution of the mixture to be analysed and *n*-octane (internal standard) are added, in an amount of 0.5–1.0 ml, to the catalyst, and the vessel is closed. After two hours reaction at room temperature, the reaction mixture is decanted off the Ni–NiS suspension and kept for GC analysis in a bottle with a rubber septum closure, which permits sampling with an injection microsyringe (Hamilton Co., Whittier, U.S.A.).

The ethylbenzene in the reaction mixture, produced by the desulphurization of thionaphthene, is determined by the internal standard technique. The percentage of

TABLE I

MODEL SOLUTIONS OF THIONAPHTHENE IN BENZENE

n = Number of determinations; T = thionaphthene content in the solution in benzene (wt.%); T^x = percentage of thionaphthene in the solution, corresponding to the portion of thionaphthene reacted (calculated by relation (1), as the average of at least three GC determinations, for $W_{(i)}$ denoting the weight of solution to be mixed with standard; $\bar{T}^x$ = average of two parallel determinations; T' = calculated thionaphthene content in the solution in benzene; d = absolute bias of the determination ($(d = T - T')$; e = relative bias of the determination ($e = 100(T - T')/T$ (%)).

n	*Ia*			*Ib*			*Ic*	
	T (wt. %)	T^x (wt. %)	$\bar{T}^x$ (wt. %)	T' (wt. %)	d (wt. %)	e (%)	*Statistical evaluation of the relation between the values of T and T^x*	
1	2.146	1.686						
			1.704	2.146	0.000	0.0		
2		1.723					Regression line, $Y = b_0 + b_1x = 0.0096 + 0.7891x$	(2)
3	1.077	0.853						
			0.852	1.068	+0.009	+0.8		
4		0.852					$b_0 = 0.0096$	
5	0.518	0.430					95% confidence limits of the constant β_0, $-0.0016 \leqq \beta_0 \leqq +0.0208$	
			0.434	0.538	−0.020	−3.9		
6		0.437						
7	0.217	0.187					$b_1 = 0.7891$	
			0.187	0.225	−0.008	−3.7	95% confidence limits of the constant β_1, $0.7780 \leqq \beta_1 \leqq 0.8002$	
8		0.187						
9	0.082	0.070						
			0.070	0.077	+0.005	+6.1		
10		0.070					$s_{yx} = 0.013$	
11	0.042	0.035					$T' = \frac{\bar{T}^x - 0.0096}{0.7891}$	(2a)
			0.035	0.032	+0.010	+24.0		
12		0.035						

TABLE II

MODEL SOLUTIONS IN BENZENE OF MIXTURES OF THIONAPHTHENE AND ZONE REFINED NAPHTHALENE

T = Thionaphthene content in the thionaphthene–naphthalene mixture (wt.%); T^x = percentage of thionaphthene in the mixture analysed, corresponding to the portion of thionaphthene reacted (calculated by relation (1), as the average of at least three GC determinations, for $W_{(i)}$ denoting the weight of thionaphthene–naphthalene mixture, in the benzene solution, to be mixed with standard); a = amount of thionaphthene weighed out in the benzene solution; T' = calculated thionaphthene content in naphthalene (as x from regression line (3); *cf.* relation (3a)). The other constants have the same meaning as in Table I.

n	*IIa*				*IIb*			*IIc*
	T (wt. %)	a	T^x (wt. %)	$\overline{T}^x$ (wt. %)	T' (wt. %)	d (wt. %)	e (%)	*Statistical evaluation of the relation between the values of T and T^x*
1	1.892	0.506	1.555					
				1.552	1.892	0.000	0.0	
2			1.550					Regression line, $Y = b_0 + b_1x = -0.00119 + 0.82097x$ (3)
3	1.578	0.343	1.326					
				1.332	1.624	−0.046	−2.9	
4			1.339					
5	1.368	0.332	1.085					
				1.088	1.326	+0.042	+3.1	$b_0 = -0.00119$
6			1.091					95% Confidence limits of the constant β_0, $-0.02566 \leqq \beta_0 \leqq 0.02328$
7	1.055	0.282	0.861					
				0.858	1.046	+0.009	+0.8	
8			0.856					
9	0.886	0.212	0.702					$b_1 = 0.82097$
				0.706	0.861	+0.025	+2.8	95% Confidence limits of the constant β_1, $0.80947 \leqq \beta_1 \leqq 0.83247$
10			0.711					
11	0.602	0.122	0.506					
				0.508	0.620	−0.018	−3.0	
12			0.510					
13	0.094	0.025	0.079					
				0.079	0.097	−0.003	−3.2	$s_{yx} = 0.022$
14			0.079					
15	0.054	0.014	0.046					
				0.046	0.057	−0.003	−5.6	$T' = \dfrac{\overline{T}^x + 0.00119}{0.82097}$ (3a)
16			0.047					

thionaphthene in the sample analysed, T^x, corresponding to the amount of thionaphthene having reacted under the conditions of the reaction, is calculated by the use of relation (1) (ref. 12):

$$T^x \text{ (wt.\%)} = \frac{W_s \; RMR_{sr} \; A'_i \; M_t}{W_{(i)} \; RMR_{ir} \; A'_s \; M_s} \cdot 100 \qquad (1)$$

where W_s is the weight of the standard added, $W_{(i)}$ is, when analysing the thionaphthene solutions, the weight of the solution to be mixed with the standard, or, in the case of solutions of the thionaphthene–naphthalene mixtures or technical grade naphthalenes, the weight of the mixture and the naphthalene, respectively, to be mixed with the standard, RMR_{sr} and RMR_{ir} are the relative molar responses of the internal standard (*n*-octane) and of the substance determined (ethylbenzene), A'_i and A'_s are the ethylbenzene and standard peak areas, and M_t and M_s are the molecular weights of thionaphthene and the internal standard, respectively. The RMR_{sr}/RMR_{ir} ratio was found to have a value of 1.066 by the internal standard technique.

The values of T^x, expressed as weight percentages, are presented in Tables Ia, IIa, and III. Each result represents the average of at least three determinations. The results of the series of determinations of thionaphthene in the model solutions of thionaphthene in benzene (Table Ia) and the model solutions of thionaphthene and naphthalene in benzene (Table IIa) have been processed by regression analysis, as described by FELIX AND BLÁHA[13]. The straight regression lines were estimated from the values corresponding to the points of the co-ordinates $[T,T^x]$; the meaning of the symbols T and T^x, as well as the respective values are quoted in Tables Ia and IIa. The

TABLE III

DETERMINATION OF THIONAPHTHENE CONTENT IN SAMPLES OF TECHNICAL GRADE NAPHTHALENE

$W_{(i)}$ = Naphthalene weighed out in the benzene solution to be mixed with standard (in grams); T^x = percentage of thionaphthene in the sample analysed, corresponding to the portion of thionaphthene reacted (calculated as the average of at least three GC determinations, for $W_{(i)}$ denoting the weight of naphthalene); T' = calculated thionaphthene content in naphthalene (as x from regression line (3); *cf.* relation (3a)); T^a = thionaphthene content calculated from the total sulphur content determined according to GRANATELLI[5]; T^b = thionaphthene content in naphthalene determined, in identical samples, by NIGAM *et al.*[10]; n = number of parallel determinations; e = difference between two parallel determinations expressed in percentage ($e = 100(T_1' - T_2')/\overline{T}'$). A = Distilled naphthalene; B = sublimed naphthalene; C = hot-pressed naphthalene; D = analytical grade naphthalene.

Sample	*n*	$W_{(i)}$ *(g)*	T^x *(wt. %)*	T' *(wt. %)*	$\overline{T}'$ *(wt. %)*	*e (%)*	T^a *(wt. %)*	T^b *(wt. %)*
A	1	2.17254	0.943	1.15				
					1.15	0.1	1.12	—
	2		0.944	1.15				
B	1	2.31571	0.681	0.829				
					0.83	1.3	0.87	0.82
	2		0.690	0.840				
C	1	2.14162	1.11	1.35				
					1.36	0.5	1.49	1.25
	2		1.12	1.36				
D	1	0.23182	0.315	0.384				
					0.39	3.1	0.43	—
	2		0.325	0.396				

estimations of regression lines (2) and (3) ($Y = b_0 + b_1x$), the 95% confidence limits for the constants β_0 and β_1, and the estimation of the standard deviations, s_{xy}, are summarized for both series investigated, in Tables Ic and IIc. The values of T' ($T' = (T^x - b_0)/b_1$) and the absolute and relative errors of the determinations are summarized in Tables Ib and IIb.

The values of T', expressing the thionaphthene content of the naphthalene samples (Table III), determined by the method suggested, have been calculated by expression (3a), using the regression line (3). Expressions (3) and (3a) are quoted in Table IIc.

Note. (a) The analytical determination proper of thionaphthene in naphthalene was preceded by a GC analysis for the presence of substances in the naphthalene studied which could interfere with the final interpretation of the peak areas A'_s and A'_t due to peak overlapping or incomplete resolution.

(b) After ten subsequent analyses of the reaction mixture, it is necessary to wait for complete elution of the accumulated naphthalene and nonconverted thio-

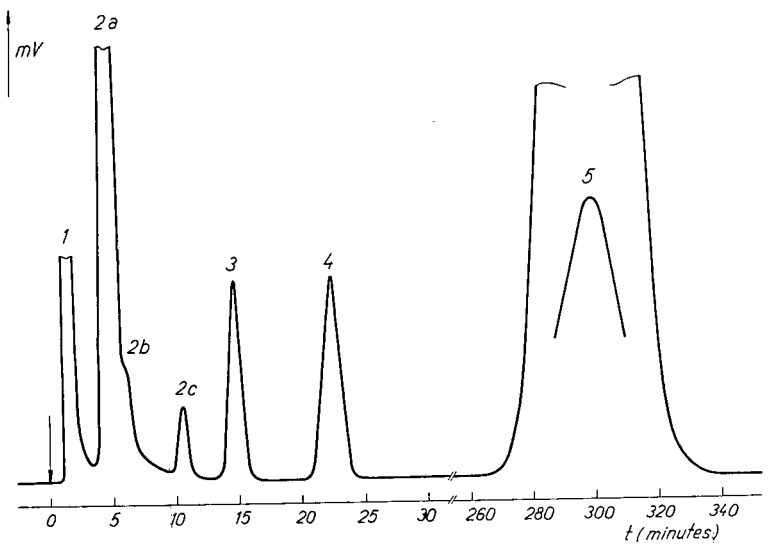


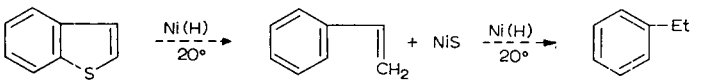
Fig. 1. Chromatographic record of an ordinary analysis of naphthalene for its thionaphthene content. 1 = Isopropyl alcohol (coming from the preparation of Raney nickel); 2a = solvent (benzene); 2b = cyclohexane; 2c = impurity in the solvent (toluene); 3 = *n*-octane; 4 = ethylbenzene; 5 = naphthalene and unreacted thionaphthene.

naphthene from the column (Fig. 1). When employing the method for routine analysis a precolumn is recommended to use for retaining the high-boiling components which can be eluted from the precolumn by back flushing or by bypass during analysis.

DISCUSSION

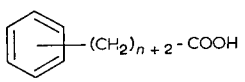
Starting from the basic mechanism of the hydrogenation–desulphurization by Raney nickel of organic sulphur compounds, as has been quoted by MOZINGO *et al.*[1],

and reckoning the amount of the hydrogen fixed to Raney nickel to be 25–150 ml/g (ref. 14), we can assume the hydrogenolysis of thionaphthene to proceed as follows:

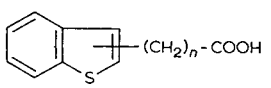


Easy hydrogenation of the conjugated double bond in styrene, the intermediate in the reaction of thionaphthene hydrogenolysis, is known[3], as is the fact that the aromatic core of styrene remains intact under such mild conditions of hydrogenation[7,15]. The quantitativeness of the course of styrene hydrogenation under the conditions employed was checked by capillary gas chromatography on a squalane stationary phase. Not even trace amounts of styrene remained in the reaction mixture after the reaction of styrene with Raney nickel was finished, and only traces of cyclohexane and of the respective alkyl derivatives resulted from the reaction of benzene, toluene, and *n*-propylbenzene. The action of Raney nickel upon ethylbenzene yielded no trace of compounds of lower molecular weight, which would have indicated a contingent consecutive cleavage of ethylbenzene.

Hence, it is possible to conclude the quantitative part of the present study by saying that ethylbenzene is the sole product of the hydrogenation–desulphurization of thionaphthene by Raney nickel at room temperature. Our findings are in agreement with the qualitative results of the investigation by BLICKE AND SHEETS[15], and, more recently, by THOMPSON *et. al.*[7] who followed the original MOZINGO procedure[1] and performed the hydrogenolysis at the temperature of the reaction medium's boiling point. BLICKE AND SHEETS only isolated products of the type



after the desulphurization of substituted carboxylic acids of the type



THOMPSON *et al.* have proved by infrared spectroscopy that the products of the desulphurization of thiaindanes consist only of the respective alkylbenzenes.

It was found that the hydrogenolysis by Raney nickel of thionaphthene in a benzene medium does not proceed quantitatively at room temperature. Therefore, we investigated the progress of thionaphthene hydrogenolysis with two model solutions of thionaphthene in benzene (thionaphthene contents were 0.1 and 1.0 wt.%) in the presence of Raney nickel charges prepared from 0.2 and 0.8 g of the alloy per 1 ml of the model solution. In 60–80 min, the portion of thionaphthene reacted had reached a limit of approximately 80%, and, over four days, it neither rose nor fell. The results of the analyses of samples taken sequentially from the model solutions within the first 4 h are depicted in Fig. 2.

The amount of the Raney nickel used for desulphurization per 1 ml of the sample analysed significantly influences the rate and quantitativeness of the reaction. The minimum amount of Raney alloy (in g per 1 ml of 0.05–2.5% solution) is quoted in Table IV. If higher amounts of Raney nickel are used, the portion of thionaphthene reacted does not rise any higher (Fig. 3).

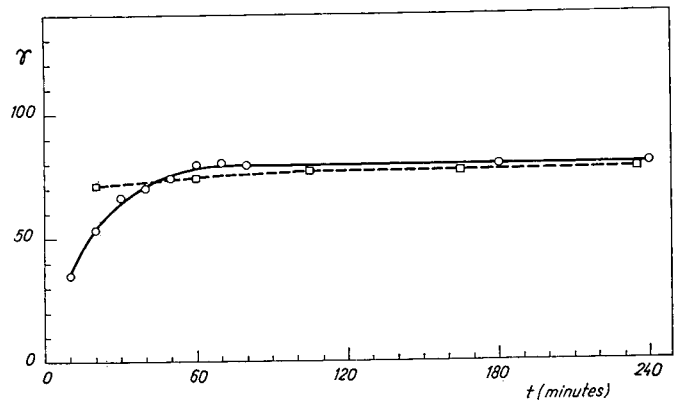


Fig. 2. Progress of the reaction of thionaphthene with Raney nickel. γ = Fraction of thionaphthene having reacted (%); t = time (min); ○ = 1% solution of thionaphthene in benzene; □ = 0.1% solution of thionaphthene in benzene.

On the basis of the above results, it is possible to recommend a standard amount of 0.6 g of Raney nickel per 1 ml of the solution to be analysed and a two hours reaction time for the desulphurization of solutions containing thionaphthene in concentrations within 0.01–0.5%, which corresponds to contents of 0.05–2.0% of thionaphthene in technical grade naphthalenes. Solutions with higher thionaphthene contents have to be diluted so that they contain not more than 2.5% of thionaphthene. At still higher thionaphthene concentrations, the amount of the Raney nickel necessary for reproducible 80% conversion would lead to the formation of a slurry.

The statistical analysis of the results shows that, with both the model series of the solution of thionaphthene in benzene (regression line (2), Table Ic) and the model series of the solutions of the thionaphthene–naphthalene mixtures in benzene (regression line (3), Table IIc), there is a linear relationship between the amount of

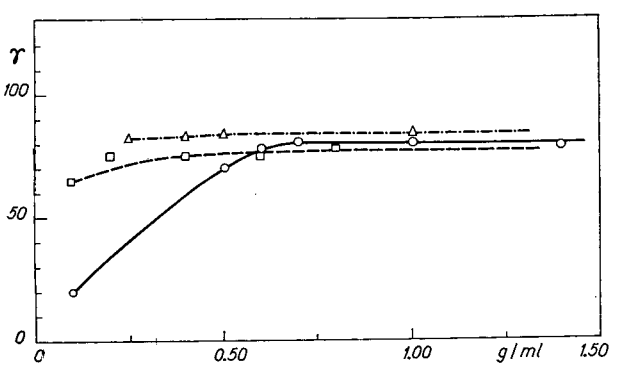


Fig. 3. Effect of the amount of Raney nickel (g/ml) on the quantitativeness of the hydrogenation–desulphurization of thionaphthene. γ = fraction of thionaphthene having reacted; ○ = 1% solution of thionaphthene in benzene; □ = 0.1% solution of thionaphthene in benzene; △ = 0.1% solution of thionaphthene in benzene (0.6% of thionaphthene in naphthalene). The individual samples were taken at two hourly intervals.

TABLE IV

MINIMUM AMOUNT OF RANEY ALLOY NECESSARY FOR REPRODUCIBLE CONVERSION OF THIONAPHTHENE TO ETHYLBENZENE

Thionaphthene content in benzene (wt.%)	*Amount of Raney alloy (in g per 1 ml of solution)*
2.5	1.8
2.0	1.4
1.0	0.8
0.5	0.6
0.25	0.4
0.1	0.2
0.05	0.1

the thionaphthene weighed out into the model solution and the thionaphthene which has reacted. Hence it follows that the presence of naphthalene in the solution has no effect on the character of the course of thionaphthene desulphurization. The systematic error is negligible at the significance level of $\alpha = 0.05$, and the difference between two parallel determinations is also insignificant at the above significance level. The error of the determination displays a so-called concentration dependence, *i.e.*, the error is dependent on the content of the substance under determination in the sample. It is possible therefore to say that hydrogenation–desulphurization of thionaphthene by Raney nickel, under the above conditions, is applicable to the quantitative determination of thionaphthene in naphthalene.

The thionaphthene content in naphthalene, T', determined according to the method suggested, can be calculated as the quantity x from straight regression line (3) and is given by expression (3a) (*cf.* Table IIc). The thionaphthene contents determined in the samples available of technical grade naphthalene are summarized in Table III. With regard to the inherent inhomogeneity of the samples of technical grade naphthalene, the data may be considered to agree well with the results of the checking analysis, carried out according to GRANATELLI[5] for the total sulphur content. Very close results were found for the thionaphthene contents also when analysing identical samples of technical grade naphthalenes B and C by hydrogenation in gaseous phase on Raney nickel[10].

CONCLUSIONS

Thionaphthene can be converted to ethylbenzene by hydrogenolysis with Raney nickel at room temperature in a benzene solution, ethylbenzene being the only quantitatively significant product of the reaction. The conversion of 0.01–2.0% solutions of thionaphthene in benzene is not complete (about 80%), however, it has been proved that both the qualitative and quantitative course of the desulphurization is reproducible even in the presence of a high excess of naphthalene. Thus, the feasibility has been demonstrated of using the hydrogenolytic reaction for routine determination of minute contents of thionaphthene (0.05–2.0%) in technical grade naphthalene. One

analysis, inclusive of the evaluation, takes about $2\frac{1}{2}$ h, 2 h of this time being taken by the desulphurization proper. The relative error of the determination does not exceed $\pm 5\%$ (Table II). The maximum attainable sensitivity of the determination is limited by the sensitivity of the GC detector used, as well as by the GC system chosen for the determination of the trace amounts of the hydrocarbon in the presence of a many-fold solvent excess.

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PHYTOCHEMISTRY OF THE *SALICACEAE*

II. THE EFFECT OF EXTRACTION PROCEDURES ON THE APPARENT FREE PHENOLIC GLYCOSIDE CONTENT OF *SALIX* SPECIES

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SUMMARY

A gas–liquid chromatography procedure was used to examine the effect of different extraction methods on the apparent free phenolic glycoside content of the bark of *Salix petiolaris* Sm. Previously reported decomposition of phenolic glycosides in plant extracts treated with lead subacetate was confirmed. Decomposition and interconversion of certain pure glycosides in commonly used extraction solvents were demonstrated and the effect of solvents on extracts of the above bark is described.

INTRODUCTION

If any examination of the distribution of phenolic glycosides in plants is to be significant for chemotaxonomic purposes, it is essential that the extraction procedures used should give reproducible results.

Several different extraction procedures have been used for the examination of the phenolic glycosides of both *Salix* and *Populus* species[1–5]. These methods vary greatly in minor details but usually involve extraction of the fresh or dried plant material with hot solvent; some include a purification step in which lead subacetate is used to precipitate unwanted components. In some cases, lead subacetate treatment may be followed or replaced by column chromatography, solvent extraction of the original extract, or both.

PEARL AND DARLING[6] reported that lead subacetate treatment changed the apparent glycoside content in extracts of *Populus* species to a varying degree, depending on the temperature and amount of lead subacetate used. They concluded that some of the glycosides found after this purification procedure are probably artifacts. PEARL AND DARLING currently employ a hot water extraction process in which lead subacetate is not used[7]. Despite this, apparent variations in the glycoside pattern of *Populus* species have been found.

THIEME[1] has suggested that lead subacetate treatment, or treatment with other bases, is undesirable in the examination of extracts of *Salix* species, but he only

mentions occlusion of the glycosides by the resulting precipitate rather than decomposition. He currently uses a column chromatographic method to purify extracts[8,9].

A recently developed gas–liquid chromatography procedure for the separation of phenolic glycosides[10] enabled a critical study to be made of some of the factors which influence the apparent free phenolic glycoside content of plant extracts.

EXPERIMENTAL

To separate and identify the phenolic glycosides, the gas–liquid chromatography procedure of BOLAN AND STEELE[10] was used. Two columns were employed in this work; 0.3% OV-1 on Chromosorb G acid washed/dimethyldichlorosilane (a.w./DMCS) 60–80 mesh and 0.5% cyclohexanedimethanol succinate (CHDMS) on Chromosorb G a.w./DMCS 60–80 mesh.

The glycoside extracts and pure glycosides were dissolved in Tri-Sil (Pierce Chem. Co.) as reported previously[10]. The plant extracts were prepared from the dried, powdered bark of *Salix petiolaris* Sm. Thin-layer chromatographic analysis was by the method of AUDETTE *et al.*[11].

Extraction procedures

Method 1. Stage A. 1 g of the bark was extracted with solvent in a Soxhlet apparatus for 8 h. Extracts were prepared with ethanol, methanol, water, acetone and ethyl acetate. The solvent was removed *in vacuo*, the residue taken up in several portions of warm water and the combined aqueous extracts were filtered and cooled. One quarter of the filtrate was evaporated to dryness in a vacuum rotary evaporator, dried overnight in a vacuum desiccator, treated with Tri-Sil and analysed by gas chromatography.

Stage B. The remaining aqueous filtrate was treated with cold lead subacetate solution until no further precipitate was formed. The mixture was filtered and the precipitate was washed with water. Hydrogen sulphide was bubbled through the combined filtrate and washings until all of the excess lead had precipitated. After filtering, the precipitate was washed with water and the combined filtrate and washings were made up to a convenient volume with water. One-third of this solution was treated and analysed in the same way as the aliquot from Stage A.

Stage C. The remaining clear, aqueous solution from Stage B, equivalent to 0.5 g of bark, was continuously extracted with ethyl acetate in a liquid–liquid extractor for 8 h. Both the ethyl acetate and the aqueous portions were evaporated to dryness *in vacuo*, dried overnight in a vacuum desiccator, treated with Tri-Sil and analysed by gas chromatography.

Method 2. 10 mg of bark were refluxed with 2 ml of solvent for 2 h. The mixture was filtered, the residue was washed with 1 ml of the same solvent and the washings and filtrate were combined. The extract was then treated and analysed in the same way as the aliquots from *Method 1*. Extracts were prepared with ethanol, methanol, water, acetone, ethyl acetate, chloroform, dichloromethane and pyridine.

Method 3. 10 mg of bark were refluxed for 1 h with 2 ml of Tri-Sil. The mixture was filtered and the marc refluxed with a further 2 ml of Tri-Sil for 1 h. The mixture was again filtered and the combined filtrates were evaporated to small volume (approx.

100 μl) in a vacuum rotary evaporator and then analysed by gas chromatography.

Effect of solvents on pure glycosides

Samples of about 0.5 mg of pure tremuloidin, populin, fragilin and grandidentatin were refluxed individually for 2 h with one or more of the solvents described in *Method 2*. The solutions were taken to dryness and dried overnight in a vacuum desiccator. The residues were then treated with Tri-Sil and analysed by gas chromatography.

RESULTS

Due to the previously reported results of PEARL AND DARLING[6] and the suggestion of THIEME[1] on the effects of lead subacetate treatment on the apparent glycoside content of *Populus* and *Salix* species, trials were run to establish whether or not this procedure was necessary and what effect, if any, it had on extracts of the dried bark of *Salix petiolaris* Sm.

Chromatograms of the dried, initial Soxhlet extract (*Method 1*, Stage A) showed picein to be the major component. Salicin was only present in the extracts of this species in relatively small proportions. Numerous other small peaks which corresponded with other previously reported glycosides[11] were also noted. However, after treatment of the extracts with lead subacetate solution and subsequent removal of excess lead (*Method 1*, Stage B) the proportion of salicin in the filtrate was found to have greatly increased. In some cases, salicin had become the major component of the extract while other components, with the exception of picein, had almost disappeared. Although disappearance of many of the components could be attributed to occlusion of the molecules by the precipitated lead salts[1], the amount of picein present in the extracts remained almost constant and rendered this explanation unlikely.

Further purification of the extracts by continuous liquid–liquid extraction with ethyl acetate was found to be undesirable in a screening procedure since, even after 12 h, glycosidic material could still be detected in the aqueous phase.

The second extraction procedure was developed in order to minimise glycoside decomposition by eliminating the lead subacetate treatment and reducing the time in which the extracts were in contact with hot solvent. Samples of bark were extracted for different lengths of time and it was found that after 2 h, extraction was virtually complete. Despite this, extracts prepared with the various solvents described *(Method 2)* showed that the number and proportions of glycosides in each extract were different (Figs. 1–4). Salicin was present in these extracts only in very small amounts and picein was always the major component. Thin-layer chromatography was used to confirm the above results and in some cases the salicin content of the extracts was so small that it could not be detected by this means. Extracts prepared with chloroform or dichloromethane showed no detectable quantities of glycosidic material.

Extracts prepared with pyridine *(Method 2)* or Tri-Sil *(Method 3)* gave similar results. The extracts contained a much larger proportion of the higher molecular weight components but later it was found that pyridine caused a substantial amount of decomposition and neither of these solvents was suitable for extraction purposes.

Extracts prepared using *Method 2* and subsequently treated with lead sub-

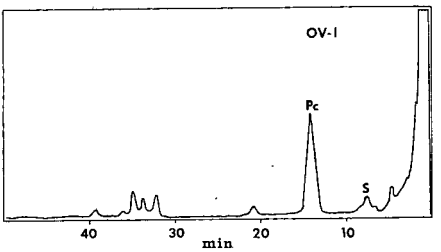

Fig. 1

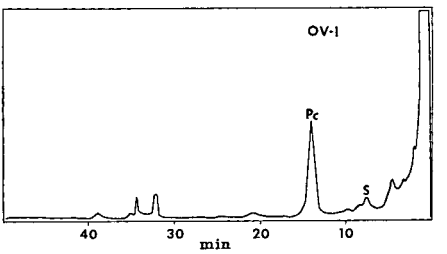

Fig. 2

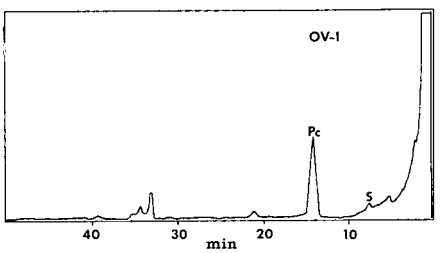

Fig. 3

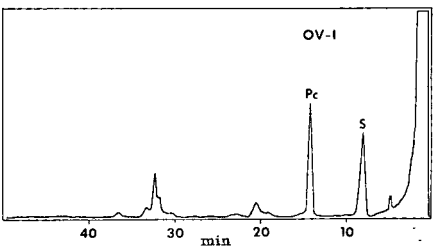

Fig. 4

Fig. 1. Gas chromatogram of ethanol extract of bark. S = salicin; Pc = picein.

Fig. 2. Gas chromatogram of methanol extract of bark. S = salicin; Pc = picein.

Fig. 3. Gas chromatogram of acetone extract of bark. S = salicin; Pc = picein.

Fig. 4. Gas chromatogram of aqueous extract of bark. S = salicin; Pc = picein.

acetate solution gave a completely different pattern of the apparent glycoside content of the extracts. Fig. 5 serves as a typical example of this effect and shows equal amounts of salicin and picein. These results confirmed that this treatment was responsible for large qualitative and quantitative changes in the glycoside portion of the extract.

Due to the variations in apparent glycoside content of extracts prepared with different solvents, a series of experiments was carried out to determine the effect of boiling solvents on pure glycosides. These tests were restricted to small quantities of a few glycosides due to the limited supply of reference compounds available.

When tremuloidin was refluxed in methanol or ethanol, gas chromatographic analysis of the residue showed that it had partially decomposed to give an unknown compound and a small amount of salicin (Fig. 6). When refluxed with water, tremuloidin was almost completely converted to populin and salicin although two other small, unidentified peaks were also noted (Fig. 7). In dimethylformamide or pyridine, tremuloidin decomposed to give large numbers of peaks, one of which corresponded to salicin, the remainder being unidentified (Fig. 8). Neither acetone nor ethyl acetate had any detectable effect on tremuloidin and refluxing tremuloidin with dichloromethane produced only traces of salicin.

After refluxing populin with water a small amount of salicin was detected in the residue and two other small peaks were also noted (Fig. 9). These two peaks

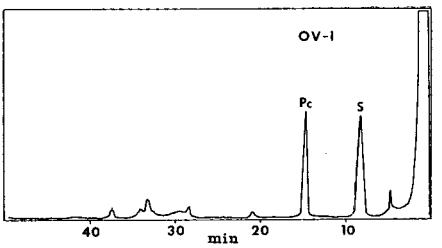

Fig. 5

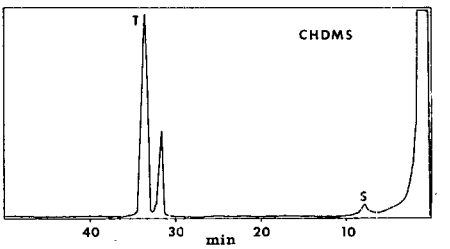

Fig. 6

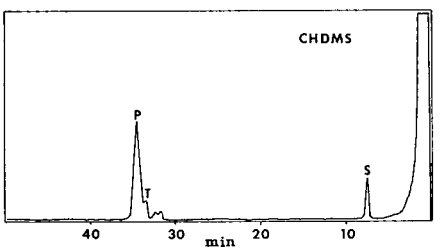

Fig. 7

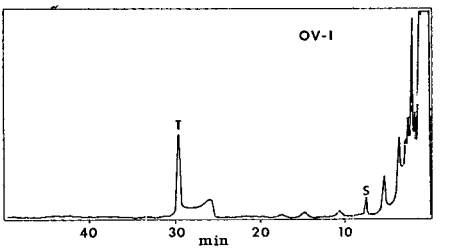

Fig. 8

Fig. 5. Gas chromatogram of ethanol extract of bark after treatment with lead subacetate solution. S = salicin; Pc = picein.

Fig. 6. Gas chromatogram of tremuloidin after refluxing with ethanol. S = salicin; T = tremuloidin.

Fig. 7. Gas chromatogram of tremuloidin after refluxing with water. S = salicin; T = tremuloidin; P = populin.

Fig. 8. Gas chromatogram of tremuloidin after refluxing with pyridine. S = salicin; T = tremuloidin.

could not be identified but they corresponded with the two small unidentified peaks formed when tremuloidin was refluxed with water (Fig. 7).

Fragilin almost completely decomposed when refluxed with water. Gas chromatograms of the residue showed that two major components were formed in almost equal proportions. One of these was salicin and the other could not be identified.

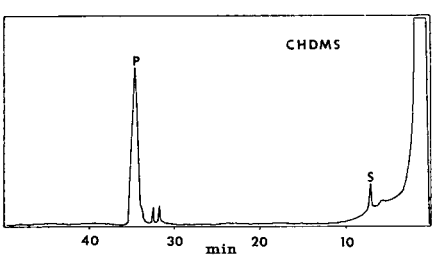

Fig. 9

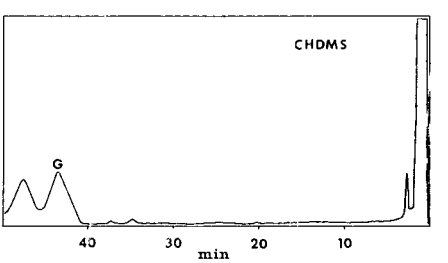

Fig. 10

Fig. 9. Gas chromatogram of populin after refluxing with water. S = salicin; P = populin.

Fig. 10. Gas chromatogram of grandidentatin after refluxing with water. G = grandidentatin.

The unidentified peak had a retention time between those of salicin and fragilin.

Some grandidentatin decomposed when refluxed with water to give several unidentified products, although a large proportion of the grandidentatin remained unchanged (Fig. 10). The major separable decomposition product was eluted shortly after grandidentatin which indicated that it probably had a molecular weight similar to the parent compound. This product may have been formed by a shift of the *p*-coumaroyl group from position 2 to position 6 on the glucose residue, similar to the shift of the benzoyl radical which occurs when tremuloidin is converted to populin[6].

DISCUSSION

The results of treating plant extracts with lead subacetate solution show that this procedure must be avoided if an accurate representation of the phenolic glycoside content of the plant is to be obtained.

It has been shown that treatment with basic substances can decompose acylated glycosides, such as tremuloidin, to form other products[2,6]. As the original extracts contained little free tremuloidin or other acylated salicin derivatives, this effect alone could not account for the large increase in salicin after lead subacetate treatment. The most likely alternative explanation for this increase is the decomposition of some unidentified glycoside complex in the extract, corresponding to the "polymeric material" suggested by PEARL AND DARLING[6], although the term "polymeric" may not be an accurate description of the nature of this unidentified component.

Since simple solvents decomposed all of the acylated glycosides tested, forming different mixtures of products, dependent upon the solvent used, it is likely that the glycoside complex in the plant material was similarly affected. This may help to explain the different glycoside patterns obtained with different extraction solvents. In addition, selective decomposition of any glycoside extracted by the hot solvent would probably enhance these differences.

These results suggest that most previous reports concerning the qualitative and quantitative phenolic glycoside content of members of the *Salicaceae* may require reinterpretation. Also, as some of the simple phenolic constituents isolated from the *Salicaceae* are known aglycones[4,12,13], some of these may not exist *per se* in the plant in the quantities reported.

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CHROM. 3951

SCINTILLATION COUNTER FOR CONTINUOUS MONITORING OF RADIOACTIVITY IN SOLUTIONS

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SUMMARY

A scintillation counter, suitable for the measurement of radioactivity in solutions irrespective of the nature of the solvent, is described. The instrument has been developed specifically for the continuous monitoring of effluents from chromatographic columns. Beads of a lithium–cerium glass, insoluble in all solvents except hydrofluoric acid, in a shallow flow-cell act as scintillator; the photons are detected with a pair of photomultipliers with coincidence circuit in conjunction with a ratemeter. The efficiency of counting ^{14}C and ^{35}S in solution is better than 20%; for ^{32}P the efficiency is about 90%, for ^{36}Cl 63%, but for ^{3}H it is only 0.3%, depending on photomultiplier and discriminator voltages and temperature selected.

INTRODUCTION

Several instruments have been described for the monitoring of radioactive isotopes by scintillation counting in effluents from column chromatography[1-4], but their use is limited to a few solvent systems because the scintillators employed are attacked by other solvents. For example, anthracene is suitable only with aqueous systems; even the use of plastic scintillators is restricted.

We have tested a lithium–cerium glass scintillator, NE 901, (Nuclear Enterprises (G.B.) Ltd.), which is insoluble in all solvents except hydrofluoric acid, and found it to respond well to ^{14}C in solution and that it could be used for the detection of ^{3}H-labelled compounds of high activity and of other isotopes. Its only disadvantage seems to be that, because of its strong phosphorescence after exposure to light, the counting cell filled with scintillator beads has to be kept in the dark for 24–48 h before a steady background counting rate can be obtained from it.

A europium-activated calcium fluoride powder gave a higher efficiency for ^{14}C than the glass scintillator and showed no phosphorescence; however, it is appreciably soluble in ammonia.

The monitor we have developed consists of a borosilicate glass optical cell 1 mm

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deep, filled with 250–300 μ diameter beads of the glass scintillator, placed between two photomultiplier tubes, in conjunction with a rate-meter suitable for coincidence counting. The output of the rate-meter is fed into a 100 mV recorder.

CONSTRUCTION OF INSTRUMENT

Photomultiplier housing and cell holder

Fig. 1 shows the individual components of the counter. Various stages of assembly are shown in Figs. 2 and 3. The two photomultiplier tubes (type 9634 QA; selected pair; E.M.I. Ltd.) are housed in a plastic pipe impervious to light (16 in. long, 2 in. I.D., Fig. 1A) with a 1/4 in. slot cut out at its middle to a depth of one-half of its diameter (Fig. 1B). The photomultipliers are pushed into the tube and are held

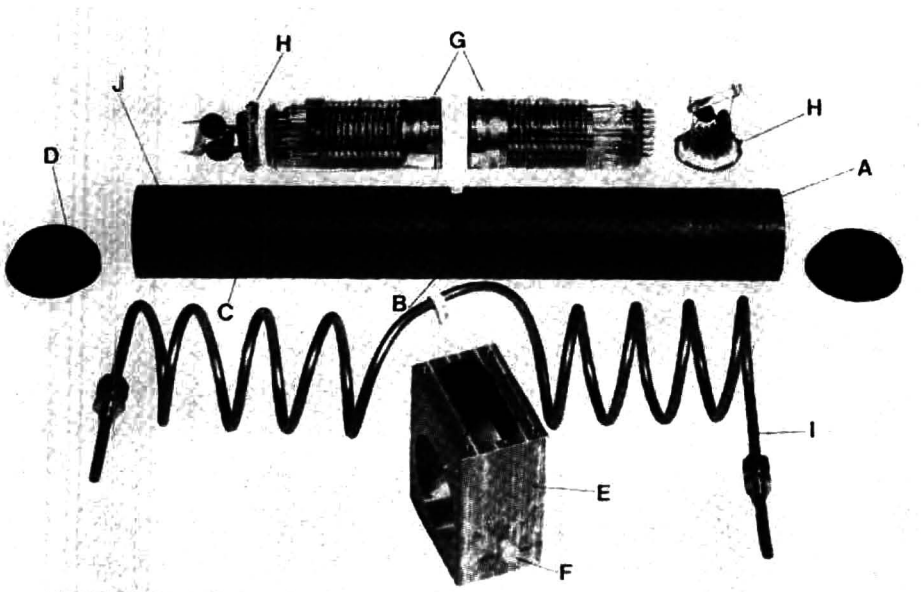

Fig. 1. Components of photomultiplier assembly: $\frac{1}{2}$ (A) Photomultiplier housing; (B) slot for counting cell; (C) tapped hole for nylon screw securing photomultiplier base; (D) plastic end-caps; (E) cell-housing; (F) nylon screw securing cell-holder to photomultiplier housing; (G) photomultiplier tubes; (H) photomultiplier connecting base; (I) cooling coil; (J) cable inlet.

in position with nylon screws tapped through the wall of the plastic holder so that their windows are level with the edge of the slot. The nylon screws locate through the fixing holes in the lugs of the photomultiplier connecting base, the lugs having been bent at right angles (Fig. 1C). The photomultiplier housing, including two recessed black plastic end-caps (Fig. 1D), is 16.7/8 in. long.

The cell-holder is made out of an aluminium block, 3 × 2 × 4 in. A hole is cut through the 3 × 4 in. faces to allow a snug fitting of the tube bearing the photomultipliers. A cavity 2.1/2 × 1 in. and 2.1/4 in. deep is then milled out of the block from one of its 3 × 2 in. faces (Fig. 1E). The cell-holder is pushed over the photomultiplier housing until the slot in the tube comes to the middle of the cavity of the

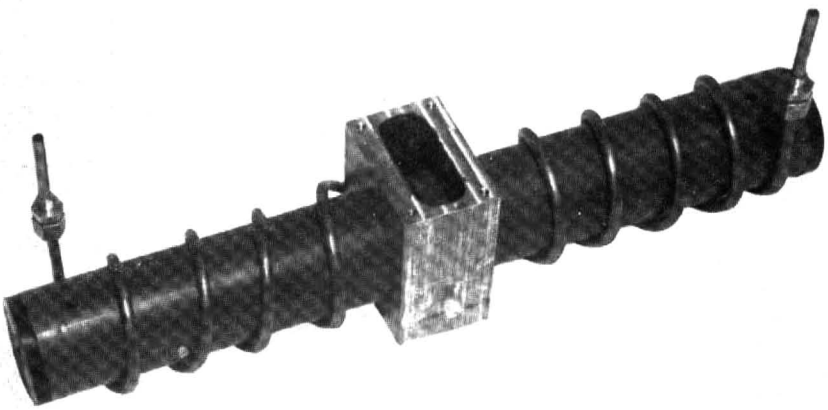

Fig. 2. Assembly of photomultiplier housing and cell-holder.

cell-holder (Fig. 2) and is secured to the photomultiplier housing by a nylon screw tapped through the cell-holder (Fig. 1F). The cell-holder is covered with a 1/2 in. recessed lid secured to the cell-holder with four screws. Two holes are drilled at an angle through the lid at one side for the connecting tubes to the counting cell (Figs. 3 and 4). A cooling coil, made of 1/4 in. O.D. copper pipe, is wound around the photo-

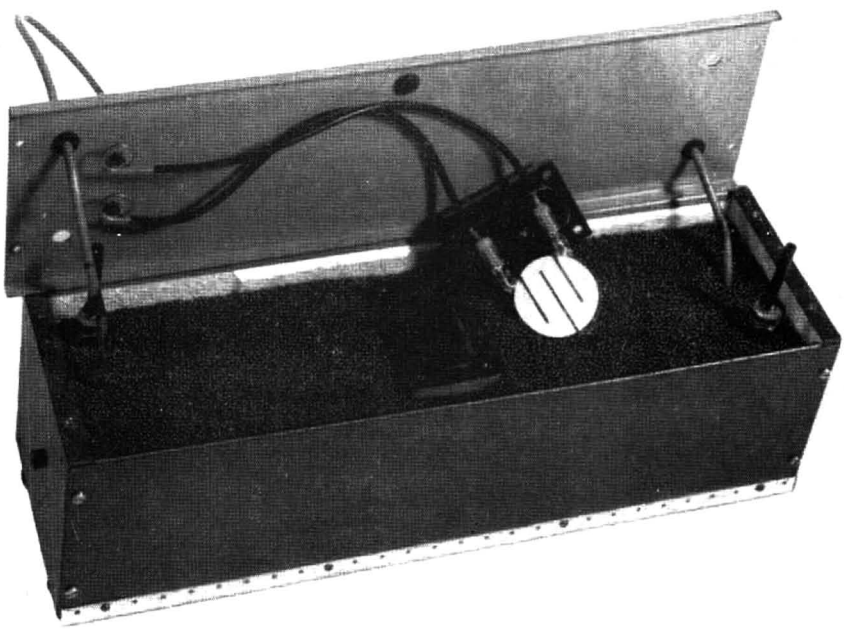

Fig. 3. Complete instrument showing counting cell in position in lid of cell-holder.

multiplier housing (Fig. 2). The whole assembly fits into a 5 × 5 × 17 in. instrument box lined with 1/2 in. expanded polystyrene (Fig. 3) and is secured to its end-plates with nylon screws tapped into the end-caps of the photomultiplier housing.

The space remaining in the box is filled with lead shot which, besides acting as a radiation shield, maintains the instrument at a constant temperature and acts as an effective light-trap.

Counting cell

The counting cell, 1 mm deep, was made of borosilicate glass by Thermal Syndicate Ltd. (London), to our design (Figs. 4 and 5). The baffle plates in the cell, fused to the optical faces, divide the cell into four compartments and direct the flow of liquid through it. The cell was at first fitted with C5 standard taper glass sockets

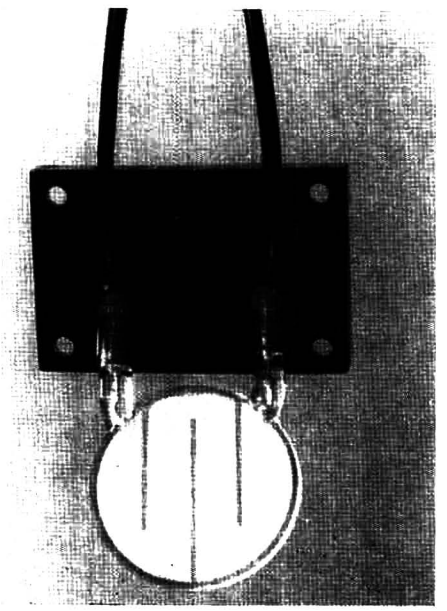

Fig. 4. Counting cell filled with scintillator beads and connected to polythene tubes through cell holder lid.

for the inlet and outlet connections; these, however, proved troublesome and were replaced with threaded glass joints. These threaded glass joints were formed over an OBA brass studding and were then fused onto the inlet and outlet ports of the cell. The locking of the flanged polythene tubes (3 mm O.D.) connecting the cell to the chromatographic column and fraction collector, into the threaded glass joints with nylon screws, is shown in detail in Fig. 5. A platinum filter (80 mesh) is clamped between the flange of the connecting polythene tube and a polythene washer on the bottom seating of the glass joint in order to prevent the washing out of the scintillator beads from the cell. The polythene tubes, covered with black PVC tubing, pass through two holes in the side of the lid to the cell-housing and are connected to two stainless steel tubes fitted onto the lid of the instrument box (Figs. 3 and 4).

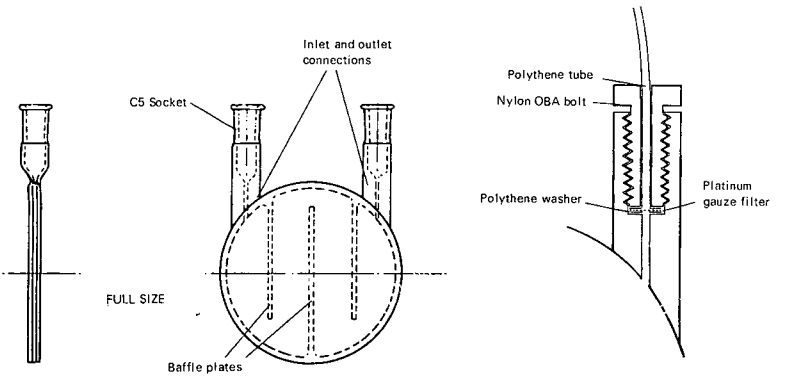

Fig. 5. Diagram of counting cell and modification to connecting ports (scales: left, 1:2; right, 1:1).

The cell is filled with the glass scintillator powder, NE 901, 250–300 μ diameter, by sucking a suspension of the powder in water through one of the ports before the platinum filter is fitted into position. The stream of water sucked through the cell carries the powder from one compartment to the other until the cell is completely filled. The powder is finally packed by drawing ethanol or methanol through the cell. After filling the cell, the wire filter is clamped into position, the cell is placed into the cell-housing, the connecting tubes are drawn through the lid of the housing and the lid is secured into position.

PERFORMANCE OF MONITOR

Fig. 6 shows the dependence of the background counting rate of the monitor on temperature after the light-induced α-phosphorescence of the scintillator has decayed. Since the radiation-induced β-phosphorescence of the scintillator is inde-

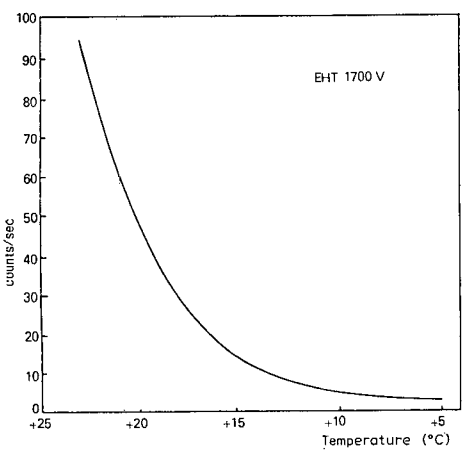

Fig. 6. Background counting rate including scintillator as a function of temperature at fixed photomultiplier and discriminator voltages (1700 and 5 V, respectively).

TABLE I

BACKGROUND COUNTING RATES (IN COUNTS PER SECOND) AT +5° AND AT VARIOUS PHOTOMULTIPLIER AND DISCRIMINATOR BIAS VOLTAGES

Bias (V)	*Photomultiplier*		
	1800 V	*1700 V*	*1600 V*
5	3.0	2.5	2.8
10	2.7	2.3	2.5
15	2.4	2.0	1.8
20	2.2	1.7	1.5
35	1.7	1.2	1.2
50	1.2	1.0	0.8

pendent of temperature, the efficiency of isotope counting is unaffected by cooling. The background counting rate at various photomultiplier and bias voltages at +5° of the tubes is shown in Table I.

The efficiency of counting ^{14}C, using a standard [^{14}C]toluene in the cell, with various bias voltages at set photomultiplier voltages is shown in Fig. 7. From the graph it can be seen that at 5°, and 1700 V, with 5 V bias, the efficiency of the monitor for ^{14}C is 21%. If, however, cooling the column effluent is unacceptable, a

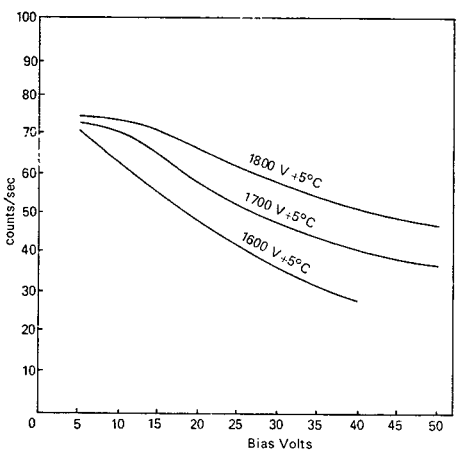

Fig. 7. Counting rates less background counts with a [^{14}C]toluene (700 disintegrations·sec^{-1}·ml^{-1}) cell volume of 0.5 ml as a function of photomultiplier voltage and discriminator bias voltage.

lower photomultiplier voltage has to be selected in order to reduce background counts at the expense of a lower efficiency of counting. A comparison of efficiencies for [^{14}C]-toluene and [^{3}H]toluene at different temperatures, with scintillator beads of varying diameters, is shown in Table II.

The NE 901 scintillator, 250–300 μ diameter beads, at room temperature and with 1100 V applied to the photomultipliers at 5 V bias, gave for various isotopes the efficiencies shown in Table III.

TABLE II

COUNTING EFFICIENCIES OF VARIOUS SCINTILLATOR BEADS WITH 1700 V APPLIED TO PHOTOMULTIPLIERS AND 5 V BIAS

Scintillator	*Temperature (°C)*	*Diameter of scintillator beads (μ)*	*Counting efficiency*	
			(%)	*Isotope*
NE 901	5	250–300	21	^{14}C
NE 901	5	125–150	30	^{14}C
NE 901	5	250–300	0.3	^{3}H
NE 901	5	125–150	0.4	^{3}H
$CaF_2(Eu)$	20 ± 2	250–300	50	^{14}C

^{125}I has been used with this monitor for column effluents but as a standardized solution was not available we were unable to calibrate the monitor for this isotope. However, a high efficiency (approximately 60%) was obtained for uncalibrated materials.

Counting [^{14}C]carbon dioxide

The cell was tested for counting ^{14}C as carbon dioxide by pumping the gas through the cell in a closed circuit. Although the scintillator gave 90–100% efficiency for $^{14}CO_2$, the system is not practical for CO_2 counting at high gas-flow rates because the cell, after filling with the scintillator beads, has a gas volume of only about 0.7 ml.

TABLE III

COUNTING EFFICIENCY OF THE NE 901 SCINTILLATOR BEADS (250–300 μ DIAMETER) AT 20 ± 2° WITH 1100 V APPLIED TO PHOTOMULTIPLIERS AND 5 V BIAS

Isotope	*Counting efficiency (%)*
^{14}C	10
^{32}P	92.5
^{35}S	10.5
^{36}Cl	62.5

USE OF THE INSTRUMENT

In practice we have been using the monitor in conjunction with chromatographic fraction collectors. The column effluent is led directly through the counting cell and then the effluent from the latter is taken to a fraction collector. The change of each collector tube is marked on the record of radioactivity, hence it is easy to identify fractions containing the isotopes. A typical record is shown in Fig. 8.

We have noted during two years use only one serious anomaly: strongly fluorescent substances (*e.g.* carotenoids) when chromatographed in light give high counting rates even though they may not contain any radioactivity. Spurious counts due to fluorescence are readily detected by the rapid decay of counts after stopping the

flow from the chromatographic column for a few seconds. Of course, radioactive fluorescent compounds can be monitored for radioactivity satisfactorily if the instrument is set up in a dark room, or if the chromatographic column and the plastic tube leading from the column to the monitor are shielded from light, *e.g.* by an envelope of black photographic paper.

"Memory" effects have been observed only with ^{125}I. When a mixture of ^{125}I-

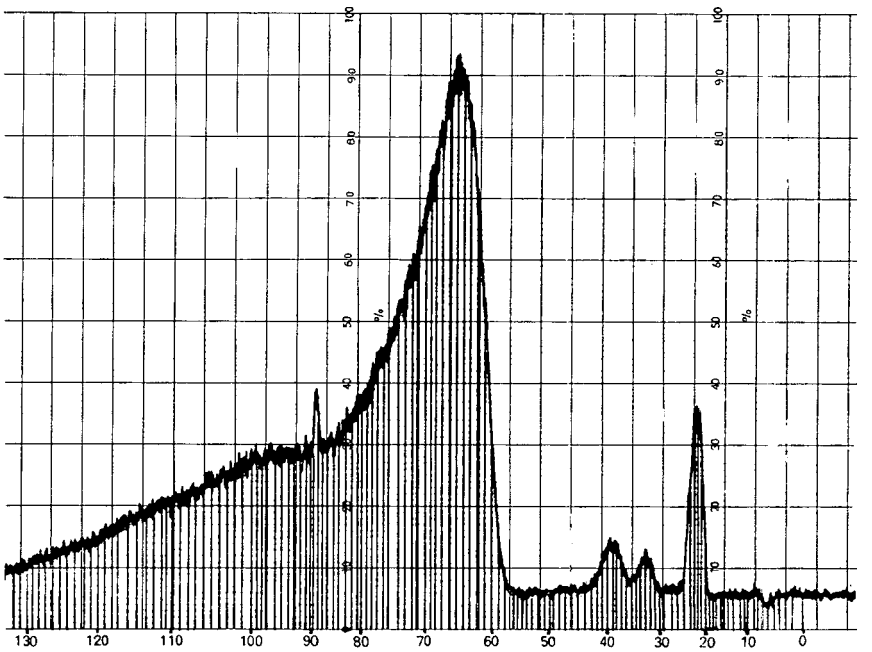

Fig. 8. Typical record obtained with instrument. This shows the separation of five fractions (^{14}C-labelled) from an incubation of yeast microsomes with farnesyl pyrophosphate (^{14}C-labelled). Chromatography of "compound X" prepared from [^{14}C]-FPP and yeast microsomes on Cellex D with 80 m*M* ammonium formate in methanol with a 0–300 m*M* NH_3 linear gradient. Counting rate: 0–100 c.p.s.; time constant: 20 sec; chart speed: 30 mm/h.

labelled iodohydrins containing a little elemental ^{125}I were chromatographed on a silicic acid column with benzene as the first solvent, the radioactive iodine was eluted with the solvent front and almost all of this remained adsorbed on the scintillator beads in the counting cell. Neither benzene nor subsequent changes of solvent to ethyl acetate–benzene mixtures shifted the iodine from the scintillator. The iodine nevertheless could be washed out very easily with methanol. The ^{125}I-labelled iodohydrins, on the other hand, were not adsorbed onto the scintillator.

In spite of these few shortcomings, the counting system described has found wide application in our laboratory mostly for detecting ^{14}C in effluents from column chromatography. It has been used with solvents such as water, ammonia, methanol, ethanol, isopropanol, ethyl acetate, hexane, benzene, chloroform, and ether.

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CHROM. 3946

RAPID COLUMN CHROMATOGRAPHIC DETERMINATION OF RIBONUCLEOTIDES IN SOUP PREPARATIONS

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SUMMARY

The authors describe a rapid and simple column chromatographic method for the determination of 5'-ribonucleotides in soup preparations by the separation and determination of the two purine bases hypoxanthine and guanine, obtained from the ribonucleotides by hydrolysis with HCl. The purines were separated on a column of a cation-exchange resin, and determined by the continuous U.V.-spectrometric monitoring of the effluent. The recovery of 5'-ribonucleotides introduced as additives into a soup preparation whose purine base content was accurately known was 100.1 ± 1.8% and 102.0 ± 1.2% on the basis of hypoxanthine and guanine, respectively.

INTRODUCTION

The current use of 5'-ribonucleotides as flavour enhancers calls for a method for their rapid analysis in foods, which can be used for production control. The literature contains various methods for the determination of ribonucleotides and the purine bases they give on acid hydrolysis. These methods are based on paper chromatography[1–9], thin-layer chromatography[10–18], and ion-exchange or gel-filtration chromatography[20–37].

Since COHN's first work[19], ion-exchange chromatography has been widely used for the separation and determination of mixtures of purines and pyrimidine bases, nucleosides, and nucleotides. The work of ANDERSON *et al.*[33] and GREEN *et al.*[36] is of particular interest in this connection because of their techniques and the resolutions obtained.

Owing to the complex nature of the mixtures to be separated, these methods involve the use of long resin columns with a low flow rate, at the expense of sensitivity. Furthermore, they are complicated and time-consuming, since the resin is generally an anion-exchanger and cannot be regenerated in the column after each analysis. To speed up the work, very high column pressures of up to 4000 p.s.i. are

sometimes used[36], in which case the usual glass columns must be replaced by stainless steel ones.

The present task was simpler, namely to separate and determine only the purine bases, and we could therefore choose a rapid, simple, and sensitive method not requiring sophisticated equipment, and which would be suitable for the routine analysis of 5′-ribonucleotides introduced into foodstuffs.

EXPERIMENTAL

Apparatus and reagents

The chromatographic column (30 × 0.9 cm) was packed to a height of 13 cm with the cation-exchange resin Aminex A5 (Bio-Rad Lab., Richmond, Calif.), the spherical particles of which had a diameter of 8–12 μ. The column was used in conjunction with a Mini-Pump (Milton Roy Co., Fla., U.S.A.) and a Beckman DB-G double-beam spectrophotometer (catalogue no. 97290) fitted with a Beckman 10 in. recorder and a microcell with a quartz window, a light path of 10 mm, and a total volume of 0.3 ml.

The reagents were 3 *N* HCl, 0.1 *N* HCl, 0.3 *M* acetic acid, adjusted to pH 3.70 ± 0.02 with 40% NaOH, and 0.3 *M* acetic acid, adjusted to pH 5.20 ± 0.02 with 40% of NaOH.

Procedure

A sample of 5–10 g was weighed out, the amount depending on the meat extract content and the yeast autolysate content. It was dissolved in 30–40 ml of water, and cooled with running water until the fats had solidified. The solids were filtered off by passing the solution through glass wool, and the filtrate was collected in a 100 ml beaker. The filtrate was then acidified to pH 1 with 3 *N* HCl and passed through the same glass wool. The filtrate was collected in a 100 ml volumetric flask, the beaker and the glass wool were washed with 0.1 *N* HCl, and the washings were also collected in the volumetric flask; the latter was then filled up to the mark with 0.1 *N* HCl.

TABLE I

RECOVERY OF 5′-RIBONUCLEOTIDES ADDED TO A SOUP PREPARATION

Sample	*Hypoxanthine added* (mg)*	*Hypoxanthine found (mg)*	*Recovery (%)*	*Guanine added* (mg)*	*Guanine found (mg)*	*Recovery (%)*
1	0.584	0.573	98.2	0.804	0.822	102.3
2	0.584	0.574	98.3	0.804	0.818	101.7
3	1.166	1.169	100.1	1.618	1.624	100.4
4	1.168	1.172	100.3	1.618	1.681	103.9
5	2.920	3.005	102.9	4.020	4.116	102.4
6	2.920	2.949	101.0	4.020	4.076	101.4
		Average recovery	100.1 ± 1.8		Average recovery	102.0 ± 1.2

* 5, 10 and 25 mg of 5′-ribonucleotides (mixture of sodium guanilate and sodium inosinate 50:50) were added to 5 g of the soup preparation corresponding respectively to 0.1, 0.2 and 0.5%.

Of this solution 20 ml were transferred quantitatively into a 30 ml ampoule, which was then sealed in a flame and kept at 121° for 2 h. The contents were transferred quantitatively into a 50 ml flask and filled up to the mark with the pH 3.70 buffer. A few millilitres of the solution were passed through a Whatman No. 42 filter paper, and 1 ml was placed on the chromatographic column. The first eluent was the pH 3.70 buffer; 27 min later, this eluent was replaced by the pH 5.20 buffer. The column temperature was kept at 55° by a thermostat.

Twelve minutes after the emergence of guanine, the wavelength of the spectrophotometer was changed from 248 mμ, while the slit was always kept at 0.5 mm.

After each analysis, the column was regenerated by washing with 1 *N* NaOH until the NaOH front had descended to three-quarters of the column, and the latter was then conditioned for 30 min with the pH 3.70 buffer.

Calculations

The following formulae were used to find the amount of 5'-ribonucleotides added to the sample from the total amount of hypoxanthine, guanine, adenine and creatinine with the aid of the data given in Table II.

$$A_m = C_r \cdot F \quad (1)$$
$$A_y = A_T - A_m \quad (2)$$
$$H_m = R_1 \cdot A_m \quad (3)$$
$$H_y = R_3 \cdot A_y \quad (4)$$
$$G_y = R_2 \cdot A_y \quad (5)$$

where:

A_T = total amount of adenine from the chromatogram
A_m = amount of adenine in the meat extract
A_y = amount of adenine in the yeast extract
H_m = amount of hypoxanthine in the meat extract
H_y = amount of hypoxanthine in the yeast extract
G_y = amount of guanine in the yeast extract
C_r = amount of creatinine determined by HADORN's method[38]
F = ratio between adenine and creatinine of the meat extract determined experimentally (see Table II)
R_1 = ratio between hypoxanthine and adenine of the meat extract determined experimentally (see Table II)
R_2 = ratio between guanine and adenine of the yeast extract determined experimentally in two different types of extract (see Table II)
R_3 = ratio between hypoxanthine and adenine of the yeast extract determined experimentally in two different types of extract (see Table II).

When the values of H_m, H_y, and G_y were calculated by eqns. (3), (4) and (5), from the total hypoxanthine and guanine given by the chromatogram one can obtain, by difference, the hypoxanthine and the guanine from the ribonucleotides added.

RESULTS AND DISCUSSION

The proposed method is rapid, accurate, and easy to carry out. The cation

TABLE II

PURINE-BASE CONTENT OF SOME MEAT EXTRACTS AND YEASTS EXTRACTS

Sample	Quality	Hypoxanthine (%)	Guanine (%)	Adenine (%)	Creatinine (%)	$F = \frac{A_m}{C_r}$*	$R_1 = \frac{H_m}{A_m}$	$R_2 = \frac{G_y}{A_y}$	$R_3 = \frac{H_y}{A_y}$
1	Meat extract	1.30	trace	0.18	6.93	0.0261	7.18	—	—
2	Meat extract	1.32	trace	0.20	8.01	0.0252	6.53	—	—
3	Meat extract	1.52	trace	0.22	7.60	0.0287	6.96	—	—
4	Meat extract	1.45	trace	0.21	7.65	0.0271	7.02	—	—
5	Meat extract	1.36	trace	0.21	7.45	0.0286	6.38	—	—
6	Meat extract	1.51	trace	0.23	7.61	0.0302	6.55	—	—
7	Meat extract	1.34	trace	0.19	7.09	0.0269	7.03	—	—
8	Meat extract	1.74	trace	0.26	7.73	0.0338	6.67	—	—
9	Meat extract	1.29	trace	0.17	7.16	0.0230	7.79	—	—
10	Meat extract	1.41	trace	0.25	7.90	0.0322	5.53	—	—
	Average values	1.42	trace	0.21	7.51	0.0281	6.76	—	—
11	Yeast extract I°	0.034	0.339	0.464	—	—	—	0.731	0.0733
12	Yeast extract	0.024	0.220	0.381	—	—	—	0.577	0.0630
13	Yeast extract	0.041	0.326	0.439	—	—	—	0.743	0.0934
14	Yeast extract	0.052	0.372	0.482	—	—	—	0.772	0.1080
15	Yeast extract	0.028	0.188	0.366	—	—	—	0.514	0.0765
16	Yeast extract	0.018	0.323	0.465	—	—	—	0.695	0.0387
17	Yeast extract	0.050	0.406	0.480	—	—	—	0.846	0.1040
18	Yeast extract	0.050	0.396	0.485	—	—	—	0.817	0.1030
19	Yeast extract	0.034	0.246	0.380	—	—	—	0.648	0.0900
20	Yeast extract	0.073	0.396	0.399	—	—	—	0.993	0.1830
21	Yeast extract	0.082	0.395	0.404	—	—	—	0.978	0.203
	Average values	0.044	0.328	0.431	—	—	—	0.756	0.103
22	Yeast extract II°	0.142	0.876	0.677	—	—	—	1.294	0.210
23	Yeast extract	0.134	0.846	0.698	—	—	—	1.212	0.192
24	Yeast extract	0.099	0.767	0.638	—	—	—	1.202	0.155
25	Yeast extract	0.098	0.833	0.688	—	—	—	1.211	0.142
26	Yeast extract	0.108	0.791	0.697	—	—	—	1.145	0.155
27	Yeast extract	0.132	0.860	0.656	—	—	—	1.311	0.201
28	Yeast extract	0.129	0.842	0.658	—	—	—	1.280	0.196
29	Yeast extract	0.141	0.877	0.664	—	—	—	1.321	0.212
30	Yeast extract	0.103	0.880	0.693	—	—	—	1.270	0.149
31	Yeast extract	0.112	0.890	0.694	—	—	—	1.282	0.161
	Average values	0.120	0.846	0.676	—	—	—	1.252	0.177

* For explanation of symbols see *Calculations*.

exchanger in a 13 × 0.9 cm column separates the purine bases very well from one another and from interfering substances (*cf.* Fig. 1). The pressure need not be more than 1 atm for the eluent to migrate down the column. The bases can be determined accurately, because they are well resolved from other substances that absorb in the U.V. region. Furthermore, the speed with which the individual bases leave the column enables one to reach a high sensitivity: even 0.02 μmole of a base can be determined very well.

To check the validity of the method, a mixture of sodium guanylate and sodium inosinate was added to the soup preparation, and the amounts of total hypoxanthine

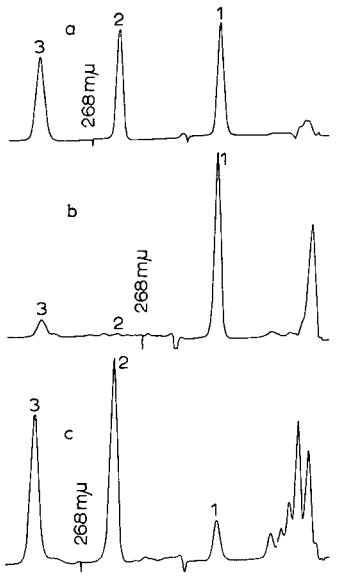

Fig. 1. (a) Chromatogram of a purine standard solution; (b) chromatogram of a meat extract; (c) chromatogram of a yeast extract. 1 = Hypoxanthine; 2 = guanine; 3 = adenine.

and guanine were determined after hydrolysis with HCl. The mixture of 5′-ribonucleotides and the soup preparation were analysed separately, so as to determine the purine base content. The results thus obtained are shown in Table I, where it can be seen that the recovery is practically quantitative, and that the method has a high accuracy.

To find the mean values of the hypoxanthine, guanine, and adenine content, many meat extract and yeast extract samples were analysed, the results being shown in Table II. This Table also gives the values of some of the ratios F, R_1, R_2, and R_3, which are used to find the amounts of 5′-ribonucleotides added to the samples from the total amount of hypoxanthine, guanine, adenine and creatinine by means of the formulae specified above.

Since the quantities of purine bases in the various meat extracts examined do not vary much, the values of the ratios F and R_1 are also fairly constant.

This is less so in the case of the yeast extracts, of which two types were analysed: the one called extract I was obtained by traditional means, while extract II was prepared by a special technique whereby a product richer in crude ribonucleotides could be obtained. These crude ribonucleotides are a mixture of guanylic and adenylic acid, in which the latter had not yet been subjected to enzymatic oxidative deamination, converting it into inosinic acid.

Inspection of the data in Table II reveals a considerable difference between the two types of autolysates as regards the values of hypoxanthine, guanine, and adenine and the values of R_2 and R_3. We think it is better to use the values for extract II (richer in 5′-ribonucleotides) for the calculations since, by using the

values for extract I, one would often run the risk of regarding some ribonucleotides naturally present in a product as additives.

These variations in the amounts of bases in the meat extracts and the yeast extracts unfortunately decrease the accuracy in the determination of the 5'-ribonucleotides introduced as additives. However if the flavour enhancer is a mixture composed of equal amounts of sodium guanylate and sodium inosinate, more reliable values can be obtained for the amounts present by carrying out checks on hypoxanthine and guanine.

On the other hand, the method is particularly useful for routine production control and for checking the guanylate and the inosinate content of a commercial mixture of 5'-ribonucleotides. In the first case, the original hypoxanthine and guanine content of the food is accurately known, and so the amount of 5'-ribonucleotides added can be determined quickly and accurately.

Determinations carried out with some commercial formulations of (50:50) 5'-ribonucleotides showed that these have a fairly constant content of hypoxanthine and guanine. The mean values used in the calculations were 11.89 and 16.08% for hypoxanthine and guanine, respectively.

CONCLUSIONS

The chromatographic method described above is rapid, accurate, and easy to perform. It is therefore useful for production control and for checking commercial mixtures of 5'-ribonucleotides. The method is also suitable for the determination of 5'-ribonucleotides added to foodstuffs, provided that a probable value is acceptable. The variation in the natural ribonucleotide content does not permit very accurate determination in such cases.

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CHROM. 3926

EXTRAKTIONSCHROMATOGRAPHISCHE TRENNUNG DER FREIEN PORPHYRINE AUF TRI-*n*-BUTYLPHOSPHAT-SÄULEN IM pH-GRADIENTEN

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SUMMARY

Separation of the free porphyrins by extraction chromatography on tri-n-butyl phosphate columns by elution with a pH gradient

The method described allows the separation of the free porphyrins on a tri-*n*-butyl phosphate (TBP) column by elution with a pH gradient. The porphyrins are eluted from the column at discrete pH values (x_D) according to the number of carboxyl groups in the side chains. Separation of isomers could not be observed under the conditions used. The x_D values indicate the centre of the pH-interval, in which the dissociation of the carboxyl groups occurs. This finding could be confirmed by spectrophotometric measurements.

By combining the method proposed with the method of separation by extraction chromatography in the TBP/1 *N* HCl partition system and the thin-layer technique in the 2,6-lutidine–water system, a very complex porphyrin mixture isolated from the urine of a patient with a hyporegenerative anaemia could be resolved. The 27 fractions obtained by these separations made clear that not only porphyrins of the reaction chain of the haem synthesis are present in the mixture, but also compounds which differ from the normal porphyrins by their substituents. However, further investigations are still necessary to elucidate the structure and determine the assumed substituents of the single fractions. In contrast to an earlier assumption it seems that porphyrins, not only of the isomeric types I and III but also of types II and/or IV, are occurring in the urine sample studied.

EINLEITUNG

Die Analyse der im Urin und Faeces ausgeschiedenen Porphyrine vermittelt einen Einblick in den Ablauf der Hämsynthese und gestattet somit Rückschlüsse auf die in pathologischen Fällen vorliegenden Störungen. Um eine eindeutige Identifizierung bezüglich des Carboxylierungsgrades und Isomerentyps zu erzielen, werden, im Hinblick auf die Vielfalt der hierbei auftretenden Hämpräkursoren, an die zur Auftrennung herangezogenen Methoden erhebliche Anforderungen gestellt. Eigene Erfahrungen mit dem an anderer Stelle beschriebenen extraktionschromatographischen Verfahren im Tri-*n*-butylphosphat (TBP)/1 *N* HCl-Verteilungssystem liessen

erkennen[1–4], dass zumindest in pathologischen Fällen die bei der Hämsynthese mittelbar bzw. unmittelbar auftretenden Metaboliten weit vielfältiger sind, als nach dem vereinfachenden, von Uroporphyrin ausgehenden Syntheseschema a priori zu erwarten war. Diese Untersuchungen machten deutlich, dass zur Analyse von Porphyringemischen nicht nur leistungsfähige Trennverfahren sondern darüber hinaus auch ausreichend strenge und zudem leicht nachprüfbare Kriterien zur Identifikation der einzelnen Fraktionen derzeit dringend erforderlich sind. Eine Bestimmung des Schmelzpunktes scheitert oft daran, dass nur geringe Substanzmengen zur Verfügung stehen und zudem bei der Präparation der Proben hohe Reinheitsforderungen gestellt werden müssen; R_F-Werte bei DC-Trennungen, die bei der Zerlegung einfacher Porphyringemische, trotz bisweilen zu beobachtender erheblicher Schwankungen im gleichen System, noch weitgehend charakteristische Kenndaten für die einzelnen Fraktionen darstellen, sind bei der Auftrennung komplexer Systeme zur Identifikation völlig unzureichend.

In der vorliegenden Arbeit wird über ein Verfahren zur Auftrennung von Porphyringemischen auf TBP-Säulen im pH-Gradienten berichtet, mit dem, in Verbindung mit der an anderer Stelle beschriebenen Methode[1–4], die Zerlegung ausserordentlich komplexer Porphyringemische durchgeführt werden kann. Während diese Methode unter den angegebenen Versuchsbedingungen eine Fraktionierung hinsichtlich des Carboxylierungsgrades ermöglicht, kann in der sich anschliessenden extraktionschromatographischen Auftrennung im TBP/1 *N* HCl-System sowie in der dünnschichtchromatographischen Zerlegung im 2,6-Lutidin/Wasser-System nach Decarboxylierung[2,3] der Isomerentyp der einzelnen Fraktionen ermittelt werden. So konnte das aus dem Urin eines porphyrieerkrankten Patienten isolierte Porphyringemisch, das dünnschichtchromatographisch in 9 Bestandteile aufspaltet[4], mit den angegebenen Methoden in 27 diskrete Einzelfraktionen zerlegt werden! Zur Identifikation der einzelnen Verbindungen, die sich hinsichtlich ihrer Intensität ausserordentlich unterscheiden, werden neben den üblichen Grössen, R_F-Wert, Retentionsvolumen $v_{max.}^{corr.}$ bzw. Retentionszeit $t_{El.}$, die Peaklage der Soretbande λ_p sowie die bei der pH-Gradientenelution auftretenden sehr charakteristischen Dissoziationsstufen x_D herangezogen.

EXPERIMENTELLER TEIL

Material

Tri-n-butylphosphat (TBP). Das zur Imprägnierung des Trägermaterials Hostaflon C 2 verwendete Extraktionsmittel TBP, ein handelsübliches Produkt (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 317), wurde ohne jegliche zusätzliche Reinigung zu den Untersuchungen eingesetzt.

Polytrifluorochloräthylen (Hostaflon C 2). Als Trägermaterial wurde die durch Aufbereitung des aus dem Handel bezogenen Produktes (Hersteller: Farbwerke Höchst AG, Frankfurt-Höchst) erhaltene Siebfraktion mit der Korngrösse von 50–100 μ verwendet. Zur Imprägnierung wurde TBP tropfenweise unter ständigem Rühren bis zu einem Gewichtsverhältnis von Hostaflon C 2:TBP = 2:1 zugesetzt.

Phosphatpuffer nach Sørensen. Zur Herstellung der 1/15 *M* Lösungen von Na_2HPO_4 bzw. KH_2PO_4 (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 6586 bzw. 4873) wurden 9.47 g Na_2HPO_4 bzw. 9.08 g KH_2PO_4 in 1 l Aqua dest. aufgelöst. Beide

Lösungen wurden jeweils in heissem Zustand mit TBP durch Schütteln vorgesättigt.

Standardpufferlösungen. Zur Absoluteichung des verwendeten pH-Meters (pH-Meter 27 der Radiometer, Copenhagen, Dänemark) wurden die Präzisionspufferlösungen S 1500 (pH bei 20°:6.881 ± 0.005) und Type S 1510 (pH bei 20°:7.426 ± 0.005) der gleichen Herstellerfirma verwendet.

Salzsäure. Zur Auftrennung der Porphyrine im TBP/1 *N* HCl-System wurde stets 1 *N* HCl verwendet, die aus Titrisolkonzentraten (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 9970/0001) angesetzt und stets in heissem Zustand mit TBP vorgesättigt wurde.

Uroporphyrin-I bzw. -III (UP-I bzw. -III). Beide Uroporphyrinisomere wurden in Form ihrer Oktamethylester durch den Handel bezogen (Hersteller: Koch-Light Laboratories Ltd., Colnbrook, England). Nach dem Verseifen in 5 *N* HCl wurden die freien Uroporphyrine im 2,6-Lutidin/Wasser-System dünnschichtchromatographisch gereinigt. Nach der Elution mit 1 *N* HCl wurden beide aus schwach saurer Lösung ($c_{HCl} \approx 0.01$ *N*) mit TBP extrahiert. Nach der Rückextraktion mit 5 *N* HCl wurde die salzsaure Porphyrinlösung mehrfach mit Benzol (5 × 5 ml) zur Entfernung anhaftender TBP-Reste ausgewaschen, auf eine Konzentration von 100 µg UP-I bzw. UP-III/ml 5 *N* HCl eingestellt und in dieser Form zu den Untersuchungen eingesetzt. Sämtliche Pipettierungen wurden mit einer Aglaspritze vorgenommen, die das bei quantitativem Arbeiten erforderliche exakte Dosieren gestattet.

Coproporphyrin-I bzw. -III (CP-I bzw. -III). Beide Isomere wurden aus UP-I bzw. UP-III durch Decarboxylierung nach der Methode von EDMONDSON UND SCHWARTZ[5] erhalten[2,3]. Die Konzentration der eingesetzten Stammlösungen schwankte, je nach Präparation, zwischen 20 und 50 µg/ml 5 *N* HCl.

Benzol p.A. Handelsübliches Produkt (Hersteller: Fa. E. Merck, Darmstadt; Kat.-Nr. 1783).

Methoden

Bestimmung der Verteilungskoeffizienten K_D für Uroporphyrin und Coproporphyrin in Abhängigkeit vom pH-Wert. Zur optimalen Einstellung der Versuchsbedingungen bei der Auftrennung der Porphyrine im pH-Gradienten ist die Kenntnis des Verlaufes der Verteilungskoeffizienten K_D erforderlich. Hieraus können Rückschlüsse auf die zu erwartenden Trennfaktoren α ($\alpha = K_D^a/K_D^b$) bzw. Auflösung β ($\beta = \Delta x_p/T_{50}$) gezogen und Voraussagen über die Durchführbarkeit einer Trennung gemacht werden.

Der Verlauf des K_D-Wertes wurde für UP-III und CP-III im Bereich von pH 4–8 bestimmt: In ein 25 ml Messkölbchen wurden 20 ml Pufferlösung (1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4) eingefüllt, 10.0 µg des betr. Hämpräkursors zugesetzt, mit 0.1 *N* NaOH auf den gewünschten pH eingestellt und schliesslich mit Aqua dest. auf 25 ml aufgefüllt. In drei mit Glasschliff versehenen Extraktionsröhrchen (100 × 16 mm; NS 14.5) werden jeweils 5.00 ml dieser Lösung einpipettiert. Nach Zugabe von 1.50 ml TBP wird für 2 min intensiv geschüttelt und anschliessend für 5 min bei 3000 U.p.m. zentrifugiert (Extraktion I). Vom Überstand (TBP) wird jeweils 1.00 ml TBP in ein gesondertes Röhrchen der gleichen Abmessungen überführt und die vorliegenden Porphyrinanteile mit 5.00 ml 5 *N* HCl rückextrahiert (Extraktion II). Nach dem Zentrifugieren wird die wässrige porphyrinhaltige Phase in ein weiteres Extraktionsröhrchen eingefüllt (Extraktion III), zur Entfernung der noch anhaftenden

TBP-Reste mehrfach mit Benzol p.A. ausgewaschen und anschliessend in eine Messküvette eingefüllt. Die Bestimmung des Porphyringehaltes erfolgte im Soretmaximum bei drei verschiedenen Wellenlängen (380, 405 bzw. 410 und 430 nm[1]) mit einem Spektralphotometer (Zeiss Spektralphotometer PMQ II mit Monochromator M 4 Q III) in der üblichen Weise. Bei allen Extraktionen wurde eine unter den gleichen Bedingungen behandelte Standardprobe mitgeführt: Hierzu wurden 5.00 ml der Ausgangslösung mit 1 *N* HCl auf pH 1–2 angesäuert und der Hämpräkursor mit 1.50 ml TBP extrahiert. Aus 1.00 ml TBP wird wiederum, wie vorangehend beschrieben, mit 5.00 ml 5 *N* HCl rückextrahiert; nach dem Waschen der wässrigen Phase mit 5 × 5 ml Benzol wird der Porphyringehalt wiederum spektralphotometrisch bestimmt. Die Berechnung der K_D-Werte erfolgte unter Beachtung der bei den Extraktionen vorliegenden Phasenvolumenverhältnisse. Zur Bestimmung des pH-Wertes werden die wässrigen Phasen nach der I. Extraktion vereinigt (drei Röhrchen) und der pH mit einem absolut geeichten pH-Meter (pH-Meter 27 der Radiometer, Copenhagen, Dänemark) gemessen.

Auftrennung der freien Porphyrine auf TBP-Säulen im pH-Gradienten. Zur Durchführung der Trennungen wurde eine weitgehend automatisierte Versuchsanordnung eingesetzt (Fig. 1), wie sie bereits in modifizierter Form zur Auftrennung der Porphyrine im TBP/1 *N* HCl-System[1-3] Verwendung fand. Kernstück dieser Anordnung ist die eigentliche Trennkolonne (Länge: L_S = 120 cm; innerer Durchmesser: D_S = 5 mm), die zusammen mit einer sog. Vorwaschkolonne (Länge: L_{VS} = 15 cm; innerer Durchmesser: D_{VS} = 15 mm) durch einen Ultrathermostaten (Hersteller: Fa. Gebr. Haake KG, Berlin) auf die gewünschte Temperatur T_S gebracht wird. Beide Säulen wurden mit dem gleichen Füllmaterial, Hostaflon C 2 mit TBP im Verhältnis 2:1 imprägniert, beschickt. Hierbei hat die Vorwaschsäule die Aufgabe, die in die Trennkolonne eintretende Elutionslösung mit TBP abzusättigen. Zur Herstellung des pH-Gradienten wird das Puffergemisch 1/15 *M* Na_2HPO_4–KH_2PO_4 verwendet: In ein vorgegebenes Volumen einer 1/15 *M* KH_2PO_4-Lösung (V_{KP}) tropft 1/15 *M* Na_2HPO_4-Lösung ein, wobei die Eintropfgeschwindigkeit der Durchflussgeschwindigkeit der Säule entspricht. Letztere wird durch die Schlauchabmessungen der Proportionierpumpe (Lieferfirma: Technicon GmbH, Frankfurt/M.), die die Elutionslösung sowohl unter leichtem Überdruck in die Vorwaschsäule eindrückt als auch von der Trennsäule unter leichtem Unterdruck absaugt, bestimmt. Der Anstieg des pH-Gradienten und damit die zur Trennung erforderliche Zeitspanne wird weitgehend durch das vorgelegte Volumen V_{KP} bestimmt (Fig. 7 und 9). Hierdurch können die Versuchsbedingungen auf einfache Weise einem gegebenen Trennproblem optimal angepasst werden. Die aus der Trennsäule austretende Elutionslösung wird durch eine Durchflussküvette (1 cm MR 1 D; Hersteller: Fa. C. Zeiss, Oberkochen) geleitet, wo kontinuierlich die Transmission der Lösung bei $\lambda_{gem.}$ = 395 nm gemessen (Zeiss-Spektralphotometer PMQ II mit Monochromator M 4 Q III) und mit einem Potentiometerschreiber (Servo/Riter II, Texas Instruments, Houston, Tex., U.S.A.) fortlaufend registriert wird. Die einzelnen Fraktionen werden in einem zeitgesteuerten Fraktionssammler aufgefangen und anschliessend in der gleichenVersuchsanordnung unter Verwendung von 1 *N* HCl als Elutionsmittel weiter aufgetrennt[2,3].

Zur Isolierung und Auftrennung von im Urin auftretenden Porphyrinen wird wie folgt vorgegangen: Der schwach salzsaure Urin (pH 1–3) von einem Volumen von 20–200 ml (je nach der Höhe der vorliegenden Porphyrinkonzentration) wird mit

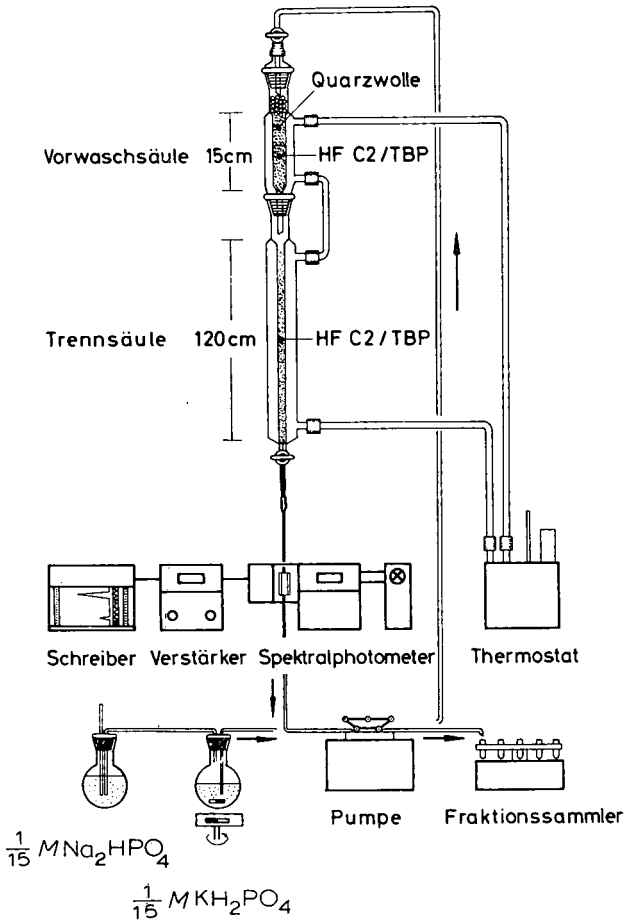

Fig. 1. Versuchsanordnung zur extraktionschromatographischen Auftrennung der freien Porphyrine durch pH-Gradientenelution mit 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4-Pufferlösung.

1–10 g imprägniertem Filterbodenmaterial[4] versetzt, kurz geschüttelt und anschliessend über eine Glasnutsche (Porenweite: G 2; Durchmesser: 50–100 mm) abgesaugt. Das Filtrat wird stets noch ein- bis zweimal über den gleichen Filterboden gegeben. Anschliessend wird mit 0.1 *N* HCl gründlich nachgewaschen. Die durch den Filterboden extrahierten Porphyrine werden nun mit 3 × 3 ml 5 *N* HCl sukzessive eluiert, die Lösungen in ein 20 ml Messkölbchen überführt und mit Aqua dest. bis zur Marke aufgefüllt. Ein Aliquot hiervon wird, nach Einstellen der Azidität mit 1 *N* NaOH auf eine Säurekonzentration von c_{HCl} = 0.01–0.1 *N*, zur Auftrennung der Porphyrine auf der Säule eingesetzt. Hierzu wird die Lösung in den Vorraum der Trennsäule eingefüllt. Bei kleinem Durchfluss (D = 10–20 ml/h) erfolgt vollständige Absorption der Porphyrine in der obersten Schicht des Füllmaterials ($K_{DB} \geqq 1000$)[4]. Anschliessend wird mit 5–10 ml 0.01 *N* HCl nachgewaschen, die Vorwaschsäule aufgesetzt und die Elution durch Einschalten der Pumpe eingeleitet. Der entscheidende

Vorzug dieser Trennmethode besteht darin, dass Porphyrine aus biologischem Material in einem einzigen Trennschritt konzentriert und ohne jegliche weitere chemische Umsetzung in freier Form unmittelbar zur Auftrennung eingesetzt werden können.

Absorptionsphotometrische Messungen. Zur Charakterisierung der bei der Auftrennung im TBP/1 *N* HCl-System erhaltenen Fraktionen wird eine Bestimmung der Peaklage λ_p der Soretbande vorgenommen. Hierzu wird der Absorptionsverlauf der salzsauren Lösungen ($c_{HCl} = 1.00\ N$) im Bereich von 380 bis 450 nm bei kleiner Spaltbreite ($S = 0.02$–0.04 mm) gemessen. Durch graphische Differentiation wird λ_p mit hoher Genauigkeit erhalten[6,7]. Zur Durchführung der Bestimmung werden Porphyrinmengen von min. 0.2 μg benötigt, um λ_p auf $\pm$ 0.1 nm genau bestimmen zu können (5 cm Mikroküvette MT 4; Hersteller: Fa. C. Zeiss, Oberkochen). Auf die Einhaltung der Säurekonzentration ist hierbei streng zu achten.

Der Einfluss des pH-Wertes auf den Verlauf der Soretbande wird für Uroporphyrin-III und Coproporphyrin-III in 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4-Pufferlösung eingehend untersucht (Fig. 4–6). Die Lage der jeweils intensivsten Bande wird, wie vorangehend beschrieben, durch graphische Differentiation ermittelt und in Abhängigkeit vom pH in Fig. 6 aufgetragen. In beiden Fällen wurden jeweils gleiche Porphyrinmengen zur Ermittlung des Absorptionsverlaufes eingesetzt.

ERGEBNISSE UND DISKUSSION

Verlauf der Verteilungskoeffizienten K_D für Uroporphyrin und Coproporphyrin

Voraussetzung für die Durchführbarkeit einer Trennung der Porphyrine auf den verwendeten TBP-Säulen ist ein ausreichend hoher Trennfaktor α. Dieser kann aus den Verteilungskoeffizienten K_D der betrachteten Hämpräkursoren unmittelbar berechnet werden.

Für die beiden physiologisch wichtigsten Porphyrine, Coproporphyrin und

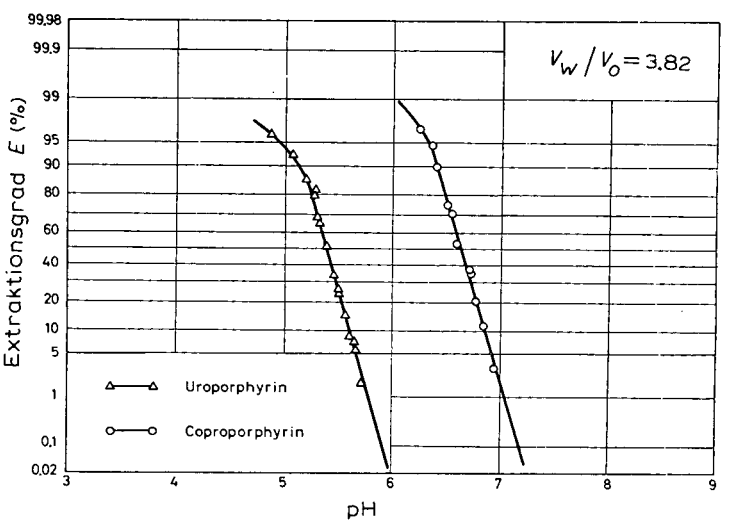

Fig. 2. Abhängigkeit des Extraktionsgrades E (Phasenvolumenquotient: $v_w/v_o = 3.84$) vom pH bei der Extraktion von Uroporphyrin und Coproporphyrin mit TBP im System TBP-1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4.

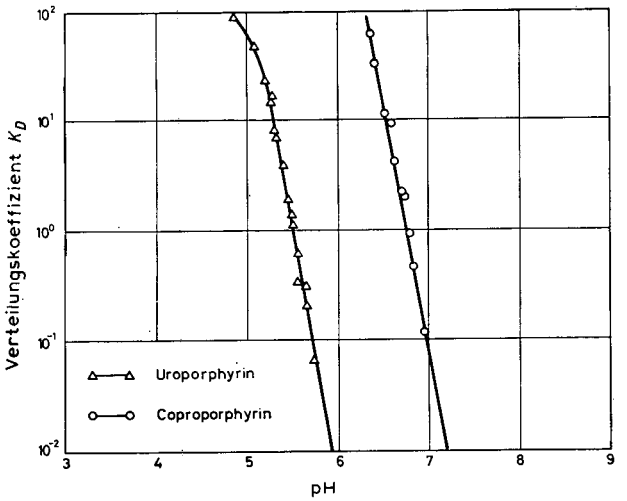

Fig. 3. Abhängigkeit des Verteilungskoeffizienten K_D vom pH bei der Extraktion von Uroporphyrin bzw. Coproporphyrin mit TBP im System TBP/ 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4.

Uroporphyrin, wurden die Extraktionsgrade E in dem interessierenden pH-Bereich zwischen 4–8 im Batch-Versuch ermittelt und unter Berücksichtigung der vorliegenden Phasenvolumenverhältnisse in die entsprechenden K_D-Werte umgerechnet. Aus dem in Fig. 2 und 3 dargestellten Verlauf geht hervor, dass in beiden Fällen die Umwandlung einer durch TBP extrahierbaren Molekül- bzw. Ionenspezies (Zustand I) in eine nicht extrahierbare (Zustand II) innerhalb eines sehr begrenzten pH-Intervalls erfolgt. So vollzieht sich die Umwandlung von 95.4% der vorliegenden Porphy-

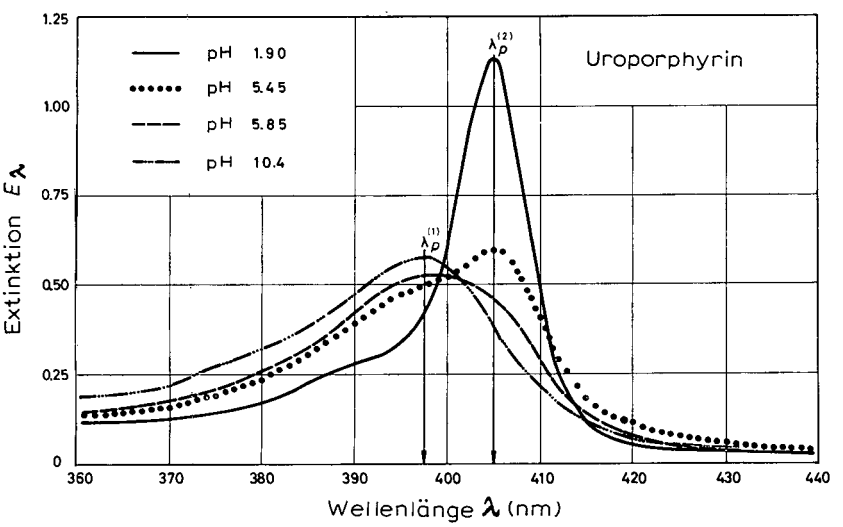

Fig. 4. Verlauf der Soretbande von Uroporphyrin bei verschiedenen pH-Werten in 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4-Pufferlösung. In allen Fällen wurde die gleiche Uroporphyrinmenge vorgegeben.

rinmoleküle von Zustand I nach Zustand II innerhalb eines pH-Bereiches von 0.8–0.9 pH-Einheiten.

Aus den K_D-Werten kann für die beiden Hämpräkursoren in dem gesamten, interessierenden pH-Bereich ein Trennfaktor von $\alpha = 10^7$–10^8 abgeschätzt werden. Somit sind für eine säulenchromatographische Auftrennung der beiden Porphyrine sehr günstige Voraussetzungen gegeben.

Verlauf der Absorptionsspektren von Uroporphyrin und Coproporphyrin im pH-Bereich zwischen 2–12

Aus dem Verlauf der Absorptionsspektren kann auf den Dissoziationszustand der Porphyrinmoleküle in den verschiedenen pH-Bereichen geschlossen werden. So wird aus der Verschiebung der Soretbande von Coproporphyrin von 402 auf 372 nm bei pH 4 bzw. von 372 auf 391 nm bei pH 7 auf das Vorliegen einer di- bzw. monokationischen Form dieses Hämpräkursors geschlossen[8]. Oberhalb von pH 7 existiert Coproporphyrin nur als freie Base, der die Soretbande bei $\lambda_p = 391$ nm zugeschrieben

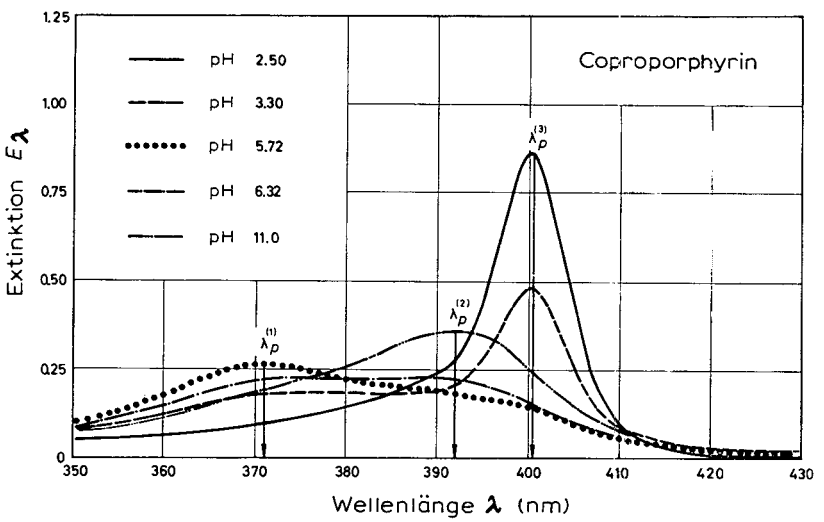

Fig. 5. Verlauf der Soretbande von Coproporphyrin bei verschiedenen pH-Werten in 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4-Pufferlösung. In allen Fällen wurde die gleiche Coproporphyrinmenge vorgegeben.

wird. Es war nun naheliegend zu prüfen, inwieweit der Dissoziationszustand dieser beiden Hämpräkursoren mit dem Extraktionsverhalten im TBP/Na_2HPO_4–KH_2PO_4-Puffersystem korreliert werden kann.

Hierzu wurden die Absorptionsspektren von Uroporphyrin und Coproporphyrin zwischen 350–430 nm im pH-Bereich von 2 bis 12 aufgenommen und die Peaklage λ_p der jeweils intensivsten Bande durch graphische Differentiation des Bandenverlaufes ermittelt. Die für beide Porphyrine erhaltenen λ_p-Werte sind in Fig. 6 in Abhängigkeit vom pH-Wert aufgetragen.

Bemerkenswert sind hierbei die in beiden Fällen auftretenden Unstetigkeitsstufen x_u, wobei für Coproporphyrin eine bei pH 4 und 6.6, für Uroporphyrin ledig-

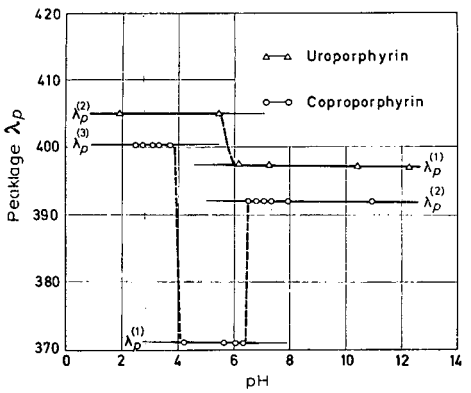

Fig. 6. Abhängigkeit der Peaklage λ_p der Soretbande von Uroporphyrin und Coproporphyrin vom pH-Wert in 1/15 *M* Na_2HPO_4–1/15 *M* KH_2PO_4-Pufferlösung. Die ausgezogenen Pfeile deuten an, dass bei der Umwandlung die zunächst intensivste Bande über die Unstetigkeitsstelle hinaus, jedoch mit abnehmender Intensität, noch zu beobachten ist.

lich eine bei pH 5.7 beobachtet werden kann. Die keilförmig ausgezogenen Pfeile (Fig. 6) sollen qualitativ andeuten, dass die zunächst intensivste Bande auch nach der Umwandlung über die Unstetigkeitsstelle hinaus mit abnehmender Intensität im Absorptionsspektrum noch nachzuweisen ist. Die Lage dieser Unstetigkeitsstellen stimmt befriedigend mit den in der Literatur angegebenen pK-Werten für die an den Stickstoffatomen von zwei Pyrrolkernen der Porphyrinmolekel eintretende Protonisierung überein[8]. Besonders auffallend jedoch ist die Übereinstimmung der x_u-Werte mit dem Mittelpunkt der Kurve der Verteilungskoeffizienten K_D ($K_D = 1$; Fig. 3), der bei der Säulentrennung der Lage der Elutionspeaks x_D entspricht. Hieraus kann gefolgert werden, dass die absorptionsphotometrisch nachweisbare Umwandlung der Porphyrinmoleküle dem Übergang einer durch TBP extrahierbaren Form in eine nicht extrahierbare entspricht. Hierbei kann im Extraktionsverhalten der beiden Hämpräkursoren, Uroporphyrin und Coproporphyrin, jedoch jeweils nur ein Übergang beobachtet werden (Uroporphyrin: pH 5.5; Coproporphyrin: pH 6.8). Es wird hierbei angenommen, dass diese Umwandlung der Porphyrinmoleküle auf eine Dissoziation der in den Seitenketten gebundenen Carboxylgruppen zurückgeht. Hierfür spricht die Tatsache, dass bei der Veresterung der Carboxylgruppen die Ester, im Gegensatz zu den freien Säuren, durch alkalische Lösung aus TBP nicht rückextrahiert werden können. Bei Einlagerung von Eisen in das Porphyrinmolekül, bei der eine Protonisierung a priori nicht mehr möglich ist, erhält man im Falle von Protoporphyrin das Hämin bzw. Hämatin, das sich im Extraktionsverhalten von dem der reinen Porphyrine nur graduell unterscheidet: Hämin ist aus alkalischer Lösung nicht, aus saurer und neutraler Suspension dagegen durch TBP ausgezeichnet extrahierbar.

Trennung der freien Porphyrine auf TBP-Säulen im pH-Gradienten

Über eine säulenchromatographische Trennung der freien Porphyrine wurde bisher in der Literatur nur vereinzelt berichtet. Bei diesen Verfahren[9–11] erfolgt, an relativ einfachen Testgemischen demonstriert, Differenzierung der einzelnen Porphyrine entsprechend des Carboxylierungsgrades. Eine Auftrennung von Isomeren wurde

hierbei nicht beobachtet. Wie an anderer Stelle bereits ausführlich beschrieben[1-3], ist es möglich, freie Porphyrine säulenchromatographisch im TBP/1 *N* HCl-System nicht nur aufzutrennen, sondern darüber hinaus auch simultan quantitativ zu bestimmen. Daneben konnten mit der beschriebenen Methode erstmals die freien Uroporphyrinisomere I und III selbst unter sehr ungünstigen Intensitätsverhältnissen aufgetrennt und ebenfalls quantitativ bestimmt werden[2].

Im Hinblick auf den sehr scharf ausgeprägten Verlauf der K_D-Werte von Uroporphyrin und Coproporphyrin (Fig. 2 und 3) war es naheliegend zu prüfen, inwieweit dieses Verhalten zur Auftrennung der Porphyrine unter diesen Versuchsbedingungen ausgenutzt werden kann. Hieraus wurden weitere Aufschlüsse über die Zusammensetzung des aus dem Urin eines porphyrieerkrankten Patienten (Pat. U.) isolierten, ausserordentlich komplexen Porphyringemisches, über das an anderer Stelle bereits ausführlich berichtet wurde[1-3], erwartet.

In Anbetracht des sehr begrenzten Umwandlungsintervalles, in dem die Dissoziation der Carboxylgruppen sich vollzieht, und der vorliegenden grossen Anzahl von Einzelfraktionen wurde die Gradientenelution einer Elution mit Lösungen diskreter pH-Werte vorgezogen. Hierbei erwies sich die in Fig. 1 dargestellte, sehr einfache Vorrichtung zur Herstellung eines (nichtlinearen) pH-Gradienten als sehr vorteilhaft: Durch Variation des Volumens der vorgelegten KH_2PO_4-Lösung (V_{KP}) konnte die Steigung des pH-Gradienten in nahezu beliebigen Grenzen variiert werden. Eine Anpassung der Versuchsbedingungen an das jeweils vorliegende Trennproblem kann hierbei in optimaler Weise vorgenommen werden. Der Einfluss von V_{KP} und damit der Steilheit des pH-Gradienten auf die Auftrennung von Uroporphyrin (UP-I/UP-III), Pentacarboxylporphyrin (5-III) und Coproporphyrin (CP-I/CP-III) kann aus den beiden in Fig. 7 dargestellten Beispielen entnommen werden. Je nach Anzahl der aufzutrennenden Komponenten kann somit die zur Trennung erforderliche Steilheit über das vorgelegte Volumen V_{KP} gewählt werden. Bemerkenswert ist bei allen in diesem System durchgeführten Trennungen, dass eine Aufspaltung in Isomere

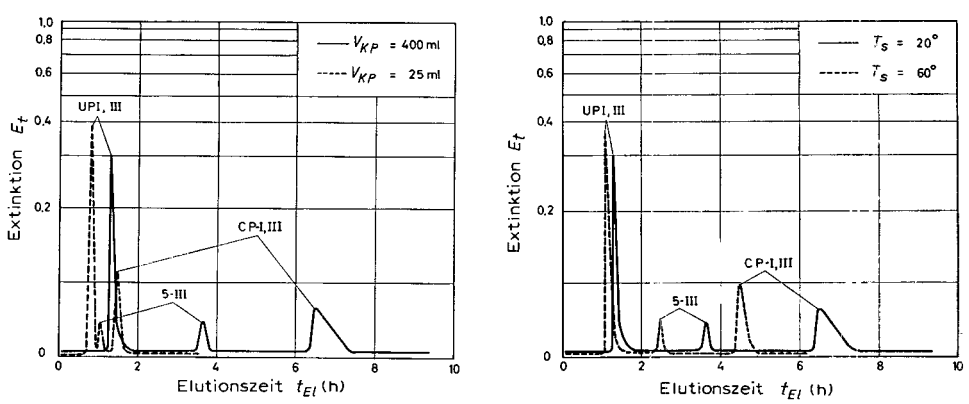

Fig. 7. Einfluss des vorgelegten Volumens V_{KP} der 1/15 *M* KH_2PO_4-Lösung auf die Auftrennung von UP-I/UP-III, 5-III und CP-I/CP-III bei konstanten Versuchsbedingungen (T_s = 20°; D = 25 ml/h; $\lambda_{gem.}$ = 395 nm.)

Fig. 8. Einfluss der Säulentemperatur T_s auf die Auftrennung von UP-I/UP-III, 5-III und CP-I/CP-III bei konstanten Versuchsbedingungen (V_{KP} = 400 ml; D = 25 ml/h; $\lambda_{gem.}$ = 395 nm).

selbst bei Gradientensteilheiten von 0.5 pH-Einheiten/h (UP-I/III) bzw. 0.1 pH-Einheiten/h (CP-I/III) nicht beobachtet werden konnte.

Der Einfluss der Säulentemperatur T_s auf die Auftrennung von Uroporphyrin (UP-I/UP-III), Pentacarboxylporphyrin (5-III) und Coproporphyrin (CP-I/CP-III) kann aus den in Fig. 8 dargestellten Beispielen ersehen werden. Im Gegensatz zur Trennung im TBP/1 N HCl-System[1-3] ist der Temperatureinfluss im vorliegenden Trennsystem nicht sehr ausgeprägt. Neben einer leichten Verkürzung der Elutionszeiten $t_{El.}$ mit zunehmender Temperatur T_s kann eine signifikante Zunahme der Auflösung β beobachtet werden[1].

Zur Prüfung der Leistungsfähigkeit des beschriebenen Verfahrens wurde eine Zerlegung des aus dem Urin einer hyporegenerativen Anämie (Pat. U.) isolierten Porphyringemisches, das bereits im TBP/1 N HCl-System aufgetrennt und analysiert wurde[2,3], vorgenommen (Fig. 9). Wie bereits nach Fig. 2 und 3 zu erwarten, tritt

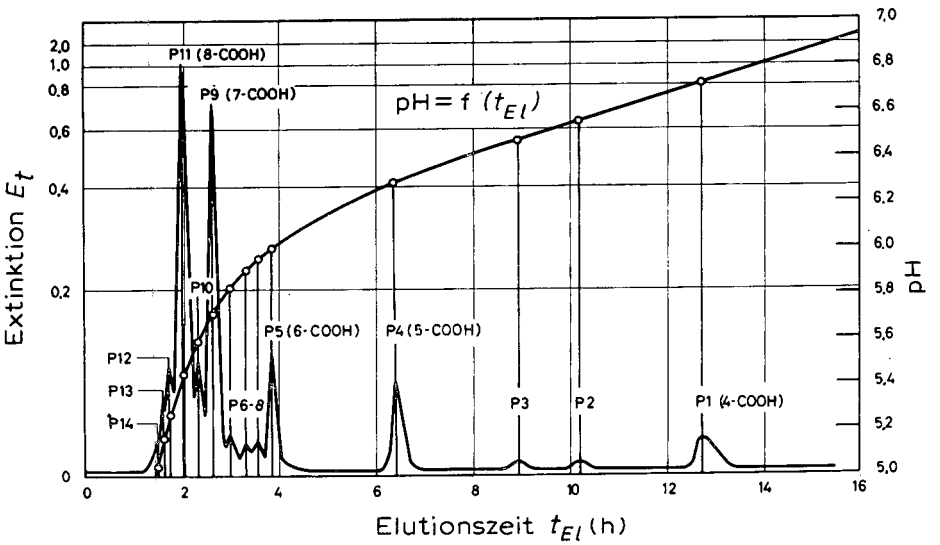

Fig. 9. Auftrennung des aus dem Urin von Pat. U. isolierten Porphyringemisches auf einer 120 cm TBP-Säule im pH-Gradienten (V_{KP} = 800 ml; T_s = 20°; $\lambda_{gem.}$ = 395 nm; D = 25 ml/h) und Ermittlung der für jede Fraktion charakteristischen Dissoziationsstufen x_D.

in diesem Trennsystem eine Umkehr der Peakfolge ein: Uroporphyrin weist die kleinere, Coproporphyrin die grössere Elutionszeit $t_{El.}$ auf. Der Verlauf des pH-Wertes der aus der Trennsäule austretenden Elutionslösung ist in der gleichen Darstellung eingetragen. Hieraus kann der jeder Fraktion entsprechende pH-Wert, der in einer grossen Versuchsreihe für die gleiche Fraktion stets innerhalb enger Grenzen (± 0.05 pH-Einheiten) reproduziert werden konnte, ermittelt werden. Die im Falle von Coproporphyrin und Uroporphyrin säulenchromatographisch erhaltenen Werte stimmen mit den im Batch-Versuch ermittelten (Fig. 2 und 3; K_D = 1) überein. Weiterhin kann im Elutionschromatogramm eine ausgeprägte Fraktionierung nach Anzahl der vorhandenen Carboxylgruppen n beobachtet werden, was leicht anhand vergleichender Untersuchungen nachzuweisen war (Bestimmung der Peaklage λ_p; Auf-

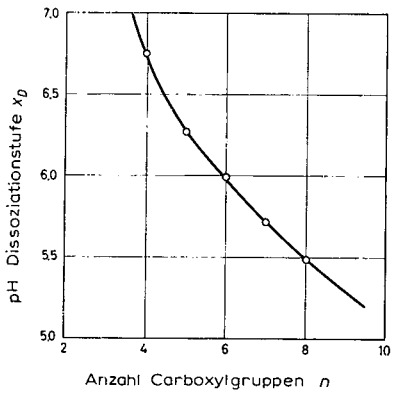

Fig. 10. Abhängigkeit der ermittelten x_D-Werte von der Anzahl der pro Porphyrinmolekül vorhandenen Carboxylgruppen n.

TABELLE I

ZUSAMMENSTELLUNG ALLER BEI DER AUFTRENNUNG DES AUS DEM URIN VON PAT. U. ISOLIERTEN PORPHYRINGEMISCHES ERHALTENEN CHARAKTERISTISCHEN KENNDATEN UNTER BERÜCKSICHTIGUNG DER BEI DEN EINZELNEN TRENNSYSTEMEN BENUTZTEN BEZEICHNUNGEN

λ_p = Peaklage der Soretbande; $t_{El.}$ = Elutionszeit bei Auftrennung im System TBP/1 N HCl; x_D = Dissoziationsstufe der einzelnen Fraktionen bei der pH-Gradientenelution; R_F = R_F-Wert bei der DC-Trennung im 2,6-Lutidin/Wasser-System. DC-Trennung im 2,6-Lutidin/Wasser-System: f; Auftrennung im TBP/1 N HCl-System: F; Auftrennung im pH-Gradienten: P.

EC/HCl	$t_{El.}$ *(h)*	λ_p *(nm)*	*DC*	R_F	*EC/pH*	x_D	*Zuordg.*
F 6	2.2	401.3	f 9	0.43	P 1a	6.75	4-III
F 6	2.2	401.3	f 8	0.40	P 1b	6.75	4-I
—	10.0	—	—	—	P 2	6.53	?
—	16.5	—	—	—	P 3a	6.45	?
—	21.0	—	—	—	P 3b	6.45	?
F 7	1.6	403.1	(f 10)	0.46	P 4a	6.27	?
F 5a	3.0	402.5	f 7	0.34	P 4b	6.27	5-III
F 5	3.6	402.3	f 6	0.29	P 4c	6.27	5-I
F 4	5.4	403.5	f 5	0.19	P 5a	5.98	6-III
F 3a	6.8	403.4	f 3,4	0.10	P 5b	5.98	6-I
F 3a	7.3	403.3	f 3,4	0.10	P 5c	5.98	?
—	—	—	—	—	P 6	5.94	?
—	—	—	—	—	P 7	5.88	?
—	—	—	—	—	P 8	5.82	?
F 2	16.0	404.6	f 2	0.05	P 9a	5.72	7-III
—	20.5	404.4	—	—	P 9b	5.72	7-I
—	24.2	—	—	—	P 9c	5.72	?
(F 3)	9.0	405.6	—	—	P 10	5.58	?
F 8	1.2	405.9	—	0.43	P 11a	5.48	?
—	3.1	405.2	—	—	P 11b	5.48	?
—	4.1	405.7	—	—	P 11c	5.48	?
(F 3)	8.8	405.9	—	—	P 11d	5.48	?
F 1a	23.5	405.7	f 1	0.02	P 11e	5.48	8-III
F 1	27.8	405.7	f 1	0.02	P 11f	5.48	8-I
—	(2.4)	(405.9)	—	—	P 12	5.28	?
—	(4.4)	(405.9)	—	—	P 13	5.12	?
—	(20.5)	(405.9)	—	—	P 14	5.04	?

trennung im System TBP/1 *N* HCl; DC-Trennung im 2,6-Lutidin/Wasser-System). Eine Aufspaltung in Isomere bei den zwischen Uroporphyrin und Coproporphyrin liegenden Hämpräkursoren (Hepta-, Hexa- und Pentacarboxylporphyrin), wie sie im System TBP/1 *N* HCl eintritt, kann nicht beobachtet werden.

Hieraus folgt, dass Porphyrine bei einer bestimmten Wasserstoffionenkonzentration eine Umwandlung erleiden, von der, wie vorangehend erläutert, angenommen wird, dass es sich um den Übergang der Carboxylgruppen der Seitenketten in die anionische Form handelt. Die erhaltenen diskreten Dissoziationsstufen x_D sind für

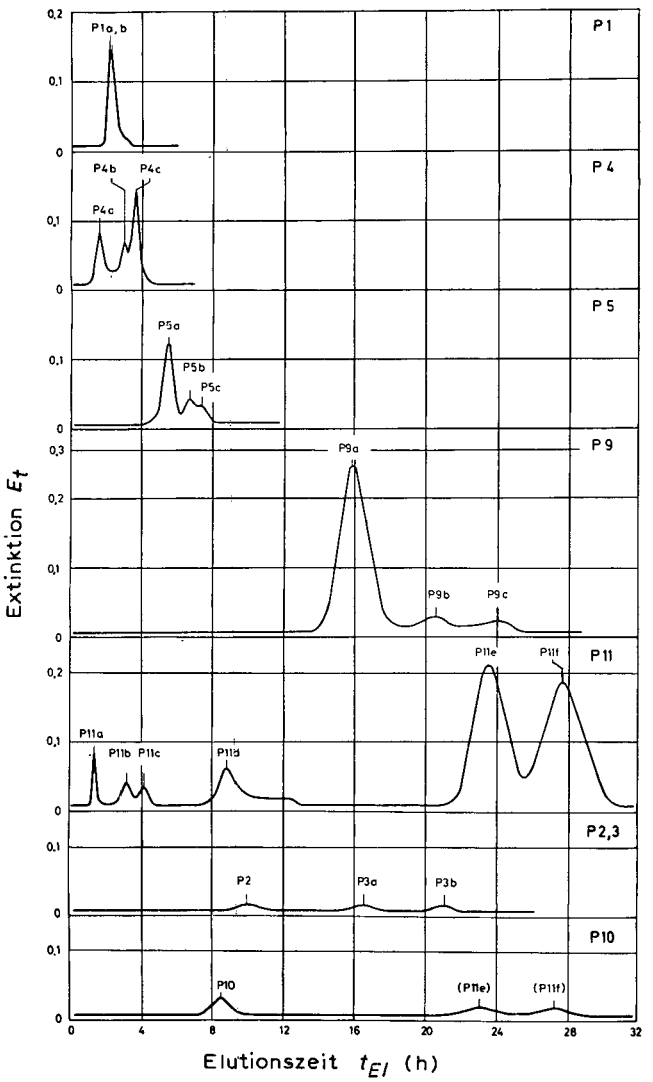

Fig. 11. Extraktionschromatographische Auftrennung der bei der pH-Gradientenelution erhaltenen einzelnen Fraktionen im Trennsystem TBP/1 *N* HCl. Bei allen Trennungen wurde die gleiche Säule (L_S = 120 cm Säule) verwendet.

jeden Hämpräkursor charakteristisch: Ihre Lage wird allein durch die Anzahl der in der Molekel vorliegenden Carboxylgruppen n bestimmt (Fig. 10). Isomere weisen bei den unter den angegebenen Versuchsbedingungen durchgeführten Untersuchungen identische x_D-Werte auf. Damit erhält man für Porphyrine mit den x_D-Werten weitere charakteristische Kenndaten, die bereits mit kleinen Substanzmengen ermittelt und somit zur Identifikation unbekannter Fraktionen herangezogen werden können. Die für sämtliche Fraktionen des aus dem Urin von Pat. U. isolierten Porphyringemisches erhaltenen x_D-Werte sind zusammen mit den bei anderen Trennungen erhaltenen Kenndaten (R_F-Werte bei DC-Trennung; x_p- und λ_p-Werte bei Trennung im TBP/1 N HCl-System) in Tabelle I zusammengestellt.

Verschiedene experimentelle Befunde (Verlauf der Peaklage bei Trennung im TBP/1 N HCl-System, vor und nach Decarboxylierung[2,3]; DC-Trennung vor und nach Decarboxylierung[2,3]) deuteten darauf hin, dass die Zusammensetzung des aus dem Urin von Pat. U. isolierten Porphyringemisches weit komplexer ist, als es nach der DC-Trennung im 2,6-Lutidin/Wasser-System (neun Fraktionen[2-4]) und der extraktionschromatographischen Auftrennung im TBP/1 N HCl-System (elf Fraktionen[3]) zunächst angenommen werden konnte. Bei der Gradientenelution treten ebenfalls eine Reihe von Fraktionen auf (Fig. 9: P 2, 3, 6, 7, 8, 10, 12, 13, 14), die sich nicht in das der Hämsynthese zugrundeliegende Schema einordnen lassen. Eine Identifikation dieser Fraktionen wurde z.T. dadurch erschwert, dass die auftretenden Konzentrationen der einzelnen Fraktionen nicht ausreichten, um weitere Umsetzungen bzw. Bestimmungen vornehmen zu können. Lediglich die bei der Auftrennung unmittelbar erhaltenen charakteristischen Dissoziationsstufen x_D sowie die in einigen Fällen ermittelten x_p-Werte im TBP/1 N HCl-System können für diese Fraktionen als spezifische Kenndaten angegeben werden (Tabelle I).

Da bei der Gradientenelution auf TBP-Säulen eine Trennung der Porphyrine entsprechend des Carboxylierungsgrades eintritt, eine Isomerentrennung nicht beobachtet wurde, war es naheliegend, die in diesem Trennsystem erhaltenen diskreten Fraktionen (P 1, 2, 3, 4, 5, 9, 10, 11) im TBP/1 N HCl-System, in dem eine Isomerentrennung möglich ist[1-4], weiter aufzutrennen. Die mit den einzelnen Fraktionen P erhaltenen Elutionschromatogramme sind in Fig. 11 zusammengestellt. Die sich hieraus ergebende Zuordnung der einzelnen Elutionspeaks in beiden Systemen, TBP/1 N HCl-System und TBP/Na_2HPO_4–KH_2PO_4-Puffersystem, kann aus der Gegenüberstellung der beiden Elutionschromatogramme in Fig. 12 entnommen werden.

Wie aus den in Fig. 11 dargestellten Beispielen hervorgeht, können die bei der Gradientenelution erhaltenen diskreten Elutionspeaks im TBP/1 N HCl-System in z.T. mehrere Fraktionen zerlegt werden. Lediglich Coproporphyrin (P 1), das dünnschichtchromatographisch im 2,6-Lutidin/Wasser-System in CP-I und CP-III aufgetrennt werden konnte[3], liess sich unter den vorliegenden Versuchsbedingungen im TBP/1 N HCl-System nicht weiter zerlegen.

Die Pentacarboxylfraktion P 4 dagegen spaltet in drei Fraktionen (P 4a, b und c), die auch bereits bei der unmittelbaren Trennung im TBP/1 N HCl-System[2,3] beobachtet wurden (F 5, 5a und 7) auf. Aus der Übereinstimmung der Peaklage der Soretbande λ_p von P 4b (λ_p = 402.5 nm) und P 4c (λ_p = 402.3 nm) folgt, dass hier die Isomere 5-I und 5-III vorliegen[2,3]. Bei P 4a handelt es sich offenbar um ein Pentacarboxylporphyrin (λ_p = 403.1 nm), das sich durch die an den Pyrrolkernen auftretenden Substituenten von den Verbindungen der Isomerenreihen I und III

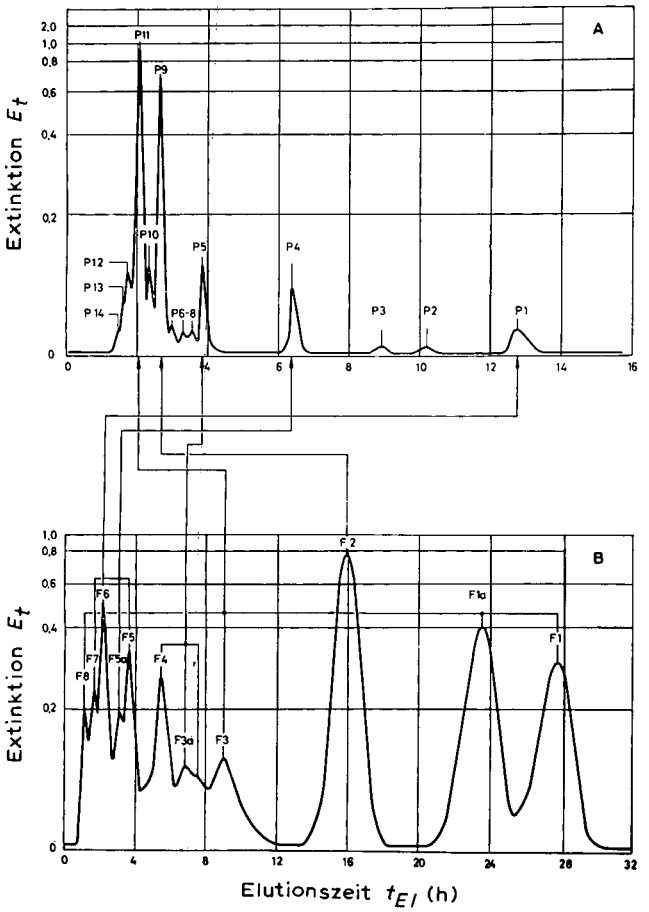

Fig. 12. Gegenüberstellung der bei der pH-Gradientenelution und im Verteilungssystem TBP/1 *N* HCl erhaltenen Elutionschromatogramme des aus dem Urin von Pat. U. isolierten Porphyringemisches sowie Zuordnung der einzelnen Fraktionen.

unterscheidet. Diese Interpretation stützt sich auf die bisher nicht bewiesene Annahme, dass alle Isomere die gleiche Lage der Soretbande aufweisen müssen. Es erscheint angebracht, diese naheliegende Annahme, die im Falle von UP-I/UP-III und CP-I/CP-III experimentell bestätigt wurde, einmal mit synthetischen Produkten bekannter Zusammensetzung und Struktur, die dem Verfasser nicht zur Verfügung standen, auf ihre Allgemeingültigkeit hin zu überprüfen.

Bei der Auftrennung der Hexacarboxylporphyrinfraktion (P 5) erhält man ebenfalls drei Fraktionen (P 5a, b und c, entsprechend F 4 und 3a[3]), deren λ_p-Werte innerhalb der Fehlerspanne der Bestimmung übereinstimmen (λ_p = 403.5 (5a), 403.4 (5b) und 403.3 (5c) nm). Hierbei weicht der für P 4b gemessene Wert von dem an anderer Stelle[3] für die gleiche Fraktion (F 3a) angegebenen Wert (λ_p = 404.7 nm) deutlich ab. Diese Abweichung wird nun dadurch erklärt, dass diskrete Fraktionen höher carboxylierter Porphyrine (P 10 u.a.) F 3a überlagert sind und somit diese

Verschiebung der Peaklage nach höheren Werten hin bewirkt[7]. Es wird daher angenommen, dass es sich, entgegen der früheren Einordnung als 7-I[2,3], um ein Hexacarboxylporphyrin der Isomerenreihe I handelt (6-I). Über die Identifikation von P 5a (= F 4) wurde bereits ausführlich an anderer Stelle berichtet[3].

Bei der Identifikation von P 5c, der bereits im ursprünglichen Porphyringemisch signifikant nachzuweisen war[3], wurde Übereinstimmung zwischen λ_p- und x_D-Werten und den für Hexacarboxylporphyrine gemessenen beobachtet. Obwohl eine starke Überlagerung von P 5b/c vorliegt, die λ_p-Bestimmung, in der rechten Flanke von P 5c durchgeführt, somit nicht sehr genau sein kann, scheinen die vorliegenden Kriterien für das Auftreten einer Hexacarboxylfraktion der Isomerenreihe II oder IV zu sprechen.

Die Zerlegung von P 9, dessen Hauptbestandteil an anderer Stelle[3] als Heptacarboxylporphyrin (7-III) identifiziert wurde, lieferte ebenfalls drei diskrete Fraktionen (P 9a, b und c), die auf das Vorliegen von Isomeren hinweisen. Während P 9b einen für Heptacarboxylporphyrine charakteristischen λ_p-Wert aufweist (λ_p = 404.4 nm), war eine solche Bestimmung für P 9c aus Intensitätsgründen nicht mehr möglich. Aus Analogiebetrachtungen kann daher gefolgert werden, dass P 9b ein 7-I-Porphyrin darstellt, eine Annahme, die durch die Übereinstimmung von λ_p-Wert und x_D-Wert mit den für Heptacarboxylporphyrine angenommenen hinreichend gestützt wird. Inwieweit es sich bei P 9c um ein weiteres Heptacarboxylporphyrinisomere handelt, müsste durch weitere Untersuchungen an grösseren Probenmengen geprüft werden.

Bei der Auftrennung von P 11 wurden neben den erwarteten Fraktionen P 11e (UP-III) und P 11f (UP-I) noch vier weitere Fraktionen mit hohen λ_p-Werten bei relativ kleinen Elutionszeiten $t_{El.}$ erhalten. Hierdurch wird das Auftreten von hohen λ_p-Werten im Bereich kleiner Elutionszeiten $t_{El.}$, für das an anderer Stelle eine befriedigende Erklärung nicht gefunden werden konnte[2,3], verständlich. Die zunächst naheliegende Vermutung, dass die sehr intensiv auftretenden Uroporphyrinisomere I und III, wegen Kapazitätsüberschreitung der Säule, bereits bei kleinen $t_{El.}$-Werten aus der Säule austreten, ist damit hinfällig. Gleichzeitig geht jedoch hieraus eindeutig hervor, dass in dem untersuchten Porphyringemisch Fraktionen auftreten, die sich nicht mehr in die vier Isomerenreihen der normalen Porphyrine einordnen lassen. Vielmehr wird, wie bereits an anderer Stelle vermutet[3], angenommen, dass diese Fraktionen sich in ihren Substituenten von den an der Hämsynthese beteiligten Porphyrinen bzw. den entsprechenden Isomeren unterscheiden. Dass es sich bei diesen Verbindungen um Porphyrinderivate handelt, kann zweifelsfrei aus dem Auftreten diskreter rotfluoreszierender Fraktionen bei der säulenchromatographischen Auftrennung und dem Vorliegen einer ausgeprägten Soretbande geschlossen werden. Zur Klärung der Struktur dieser Verbindungen und Ermittlung der Substituenten sind jedoch weitergehende Untersuchungen erforderlich, die über den Rahmen der vorliegenden Arbeit hinausgehen.

Die neben den normalen n-Carboxylporphyrinen (n = 4, 5, 6, 7 und 8) auftretenden Fraktionen (P 2, 3, 6, 7, 8, 10, 12, 13, 14) wurden z.T. (P 2, 3 und 10) extraktionschromatographisch im System TBP/1 N HCl aufgetrennt (Fig. 11). Weitergehende Untersuchungen zur Konstitutionsaufklärung waren aus Intensitätsgründen nicht möglich. Auch hier wird, wie vorangehend angedeutet, angenommen, dass es sich um Porphyrine mit abnormaler Substitution in den Seitenketten handelt.

ZUSAMMENFASSUNG

Zur Zerlegung komplexer Porphyringemische wird ein säulenchromatographisches Verfahren beschrieben, das eine Trennung der freien Porphyrine entsprechend des Carboxylierungsgrades auf TBP-Säulen im pH-Gradienten durchzuführen gestattet. Die den Elutionszeiten $t_{El.}$ entsprechenden Dissoziationsstufen x_D stellen charakteristische Kenndaten dar, die zur Identifizierung der einzelnen Porphyrinfraktionen herangezogen werden können. Die durch die x_D-Werte gekennzeichnete innermolekulare Umlagerung bzw. Dissoziation der Carboxylgruppen, die absorptionsspektrophotometrisch anhand einer Verschiebung der Soretbande nachgewiesen werden kann, vollzieht sich innerhalb eines sehr engen pH-Intervalls (95.4% in 0.8–0.9 pH-Einheiten), worauf die relativ hohe Auflösung der Peaks in diesem Elutionschromatogramm zurückzuführen ist. Eine Auftrennung von Isomeren konnte selbst bei Gradientensteigungen von 0.1 pH-Einheiten/h bzw. 25 ml bisher nicht beobachtet werden.

Durch Kombination der beschriebenen Methode mit den an anderer Stelle angegebenen Verfahren (Extraktionschromatographische Auftrennung im TBP/1 *N* HCl-System[2,3]; DC-Trennung im 2,6-Lutidin/Wasser-System) kann eine weitgehende Zerlegung selbst sehr komplexer Porphyringemische erzielt werden. So konnte das aus dem Urin von Pat. U. isolierte Porphyringemisch, das bei der DC-Trennung in neun, bei der EC-Trennung im TBP/1 *N* HCl-System in elf diskrete Fraktionen aufspaltete, durch Kombination der drei Verfahren in mind. 27 Fraktionen zerlegt werden! Eine Identifikation der einzelnen Porphyrine, die z.T. nur in sehr geringen Konzentrationen vorlagen, wurde anhand der gemessenen x_D-, λ_p-, x_p- und R_F-Werte vorgenommen. Hierbei liess sich ein grosser Teil der aufgefundenen Verbindungen nicht in das der Hämsynthese zugrundeliegende Schema einordnen. Es wird daher angenommen, dass in dem untersuchten Porphyringemisch von Pat. U. Verbindungen auftreten, deren Konstitution durch Substituentenaustausch sich von der normaler Porphyrine unterscheidet. Entgegen einer früheren Annahme kann nach den nun vorliegenden Ergebnissen das Auftreten der Isomeren von Typ II und/oder IV neben den bereits nachgewiesenen (Typ I und III) nicht ausgeschlossen werden. Zur endgültigen Bestätigung dieser Annahme sind jedoch weitergehende Untersuchungen, insbesonders solche mit synthetischen Produkte, zur Gewinnung von dringend benötigten charakteristischen Kenndaten (z.B. λ_p-, x_D- und x_p-Werte) erforderlich.

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GAS CHROMATOGRAPHIC AND COLORIMETRIC DETERMINATION OF ESTRADIOL MONOESTERS IN OIL SOLUTIONS AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of estradiol monoesters in oil solutions, for pharmaceutical use (concentration 2 mg/ml). The separation of the steroid is performed by means of thin-layer chromatography followed by a colorimetric or a gas chromatographic method for quantitative analysis. As examples, the procedure for the analysis of estradiol-17β-cyclopentylpropionate (1) and estradiol-3-benzoate (2) is reported. The TLC step is similar in both cases; in example (1) a saponification technique is applied to the steroid ester adsorbed on the silica gel thus achieving simultaneous elution and hydrolysis to estradiol, which is then extracted with ethyl ether. In example (2) a simple elution with chloroform is performed. The estradiol (example 1) or its monoester (example 2) is transformed into the respective trimethylsilyl ether (TMSE) prior to gas chromatography. For this step a method previously described by the authors for diesters of estradiol or estrone esters is employed. The colorimetric analysis is performed by BROWN's method[2] as described for urinary estrogens, with the correction of the absorption values according to ALLEN[3]. Recovery values and the precision of both methods are reported.

INTRODUCTION

In a previous paper[1] we described the separation of some derivatives of estrone and estradiol in oil solutions, for pharmaceutical use, at a concentration of 2 mg/ml by means of thin-layer chromatography (TLC).

This separation, verified quantitatively for solutions of estrone-3-benzoate and estradiol-3,17-dipropionate, allows the determination of the estrogens by two different methods; *viz.*, a colorimetric one using the reaction of sulfuric acid and hydroquinone according to BROWN[2], with correction of the spectrophotometric readings according to ALLEN[3], and a gas chromatographic (GLC) one described by us. The latter, an accurate and sensitive method, is convenient for laboratories equipped for gas chromatographic analysis, being more rapid and specific for all estrogens tested so

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far, than the colorimetric one, has recently been studied by us with the aim of making its application more general.

In our previous work we studied completely esterified estrogens, which can be directly analyzed by GLC after separation by TLC, because of their stability in the working conditions. In this paper two different separation methods with different, simple elution or elution–saponification steps, and subsequent protection of the hydroxyl are described for two monoesters of estradiol (in the 3 and 17 position), estradiol-3-benzoate and estradiol-17β-cyclopentyl propionate. These methods can also be applied to other similar esters. In particular, the elution–saponification method permits the analysis of different esters of estradiol by the same gas chromatographic method.

EXPERIMENTAL

Reagents

Ethyl ether and ethanol were purified as described by us previously[1]; hexane (benzine for chromatography b.p. 65°), methanol, chloroform, carbon disulfide were of analytical grade (a.g.). Sulfuric acid, acetic acid, hydroquinone and benzyl alcohol were also a.g.; olive oil for pharmaceutical use was refined and deacidified. Materials for TLC were Merck Silica Gel G, washed for 24 h with chloroform a.g. in a Soxhlet; this material was also used in some cases mixed with 0.3% Dupont Luminescent Chemical 609.

Reference compounds: Estradiol-17β, estradiol-3-benzoate, estradiol-17β-cyclopentylpropionate, 5α-cholestane, 5α-cholestane-3β-ol propionate (cholesteryl propionate) were analytical grade and if necessary recrystallized. Materials for gas chromatography were 80–100 mesh Gas-Chrom Q and SE-30 (methylsiloxane polymer).

Preparation of the solutions for analysis

(a) Reference solutions. The steroids to be analyzed were dissolved in ethanol at a concentration of 0.8 mg/ml and diluted to 5 and 8 μg/ml as necessary.

(b) Solutions in oil. These were prepared in a tared 10 ml glass stoppered cylinder by dissolving 8 mg of steroid in 0.1 ml of benzyl alcohol and adding olive oil to a volume of 4 ml; this solution of steroid in oil (concentration 2 mg/ml) was diluted with heptane to a final volume of 10 ml (concentration of the steroid 0.8 mg/ml, concentration of oil 0.4 ml/ml: solution b_1).

(c) Blank (oil only). A solution of oil in heptane at a concentration of 0.4 ml/ml was prepared.

(d) Standard solutions. Internal standards for GLC were prepared in heptane.

Procedure for the isolation and determination of steroids

(1) Estradiol-17β-cyclopentylpropionate

(A) Chromatographic separation. A thin-layer separation on Silica Gel G plates was performed, thickness of layer 0.5 mm, solvent hexane–ethyl ether–acetic acid (HEAA) (80:20:1), with continuous development for 3 h according to the technique described by CAVINA AND MORETTI[4]. Of the solution in heptane b_1 50 μl (40 μg steroid andapproximately 20 mg oil), were applied with a Hamilton microsyringe in a line

of 6 cm length. The steroid was identified by spraying a central lane (on which 8 μl of the same solution b_1 had been applied) with Folin-Ciocalteu's reagent (1:5) (ref. 5) and with 4 *N* NaOH. For details of the chromatographic analysis see the preceding papers[1,4].

(B) Preparation of the derivative for gas chromatographic analysis. After identification of the steroid band the corresponding silica gel (approximately 2 × 7.5 cm) was scraped off and transferred into a 30 ml glass stoppered test tube and the saponification of the estrogen ester was carried out with 4 ml 10% KOH (w/v) in methanol, by refluxing in a boiling water bath for 30 min. After cooling, 3 ml of 2 *N* HCl, 3 ml of water and 6 ml of purified and freshly distilled ethyl ether were added to the tube. After shaking, the ether phase was sucked off with a pipet and transferred into a 25 ml separator equipped with a teflon stopcock. Four successive extractions each with 2 ml ether were carried out. The collected ether extracts were washed with 2 ml of 2.5% (w/v) $NaHCO_3$ and twice with 2 ml water. The ether phase was dehydrated by passage through anhydrous sodium sulfate and filtered into a 25 ml volumetric flask. The sample was brought to dryness by evaporating the ether under a stream of nitrogen, 0.5 ml methanol was added and made up to volume with chloroform (solution C_p). Of the solution C_p 5 ml were transferred into a glass stoppered test tube and taken to dryness; 1 ml (8 μg) of the standard solution (d) of 5α-cholestane was added to the residue, the solvent was again evaporated and the residue was left overnight under vacuum in a desiccator over KOH and silica gel. In order to prepare the trimethylsilyl ethers (TMSE) according to BOUGHTON *et al.*[6], 0.1 ml of the appropriate reagent (anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane, 9:3:1) was added to the dried residue. The tube was well stoppered and left for 1 h at room temperature, then the sample was evaporated to dryness under a stream of dry nitrogen, the walls of the tube were washed with a small amount of carbon disulfide, the solution was again evaporated and the residue was dissolved in the tip of the tube in 40 μl of the same solvent. For each analysis a quantity of 2 μl was injected into the gas chromatograph.

(C) Gas chromatographic analysis. The instrument was a Perkin-Elmer model 801 gas chromatograph equipped with a flame ionization detector and a 5 mV recorder. A 2.20 m helical glass column, I.D. 2.5 mm, packed with 2% SE-30 on 80–100 mesh Gas-Chrom Q was used. The column was preconditioned as described earlier[1]. The temperatures of the oven, injector and detector were 190°, 260° and 200°, respectively. The nitrogen flow rate was 40 ml/min. The attenuation was set at × 5. For the calibration curve aliquots of 2.5, 5.0 and 7.5 μg of estradiol-17β standard were transferred, in duplicate, into conical tubes, 8 μg (1 ml) of 5α-cholestane (internal standard) were added and the samples were evaporated to dryness. The trimethylsilylethers were prepared as described before for the sample under (B). Their value $R = A/B \cdot C/D$ was calculated according to CELESTE AND TURCZAN[7] where A = peak height of analyzed steroid, B = peak height of internal standard, C = μg of internal standard and D = μg of analyzed steroid. The average R value determined was ($m \pm$ S.D.) 1.620 ± 0.006. The estradiol-17β-cyclopentylpropionate value was calculated by multiplying the estradiol found by 1.46.

(D) Colorimetric analysis. After evaporation to dryness of 6.5 ml of the solution C_p, the reaction with sulfuric acid (60% v/v) and hydroquinone as described by BROWN[2], and the correction of the absorption values according to ALLEN[3], was

used for the determination. The measurements were carried out on a Beckman DU-2 spectrophotometer at the wavelengths of 476, 514 and 552 nm.

The calibration curve was prepared with estradiol-17β; the value found was calculated for estradiol-17β-cyclopentylpropionate using the factor mentioned above.

(2) Estradiol-3-benzoate

(A) Chromatographic separation. The procedure was analogous to that described for estradiol-17β-cyclopentylpropionate. The solvent was hexane–ethyl ether–acetic acid (50:50:1) with continuous development for 1 h and 30 min.

The identification of the compound examined was performed under U.V. light (0.3% Dupont Luminescent Chemical 609 was incorporated in the silica gel).

(B) Preparation of the derivative for gas chromatographic analysis. After identification of the steroid band, the corresponding silica gel (approximately 2 × 7.5 cm) was transferred into a small tube, 0.8 cm in diameter, plugged at the bottom with a small layer of defatted cotton, and was eluted with 25 ml of chloroform (solution C_p). The preparation of the 17β-trimethylsilyl ether of estradiol-3-benzoate was performed with 5 ml of solution C_p which had been taken to dryness in a glass stoppered conical tube. The procedure was the same as in the example described above. A solution of cholesteryl propionate in heptane at a concentration of 5 μg per 1 ml was used as internal standard.

(C) Gas chromatographic analysis. The instrument and column were the same as described for estradiol-17β-cyclopentylpropionate analysis. The temperatures of the oven, injector and detector were 235°, 260° and 235° respectively; the nitrogen flow rate was 40 ml/min; the attenuator was set at × 5. For the calibration curve aliquots of 4, 8, 12 μg of estradiol-3-benzoate were used, in duplicate, and 5 μg (1 ml) of cholesteryl propionate were added. The trimethylsilyl ethers were prepared and their R value was determined as described above. The average R value was 0.683 ± 0.017.

(D) Colorimetric analysis. The procedure was the same as described before for estradiol-17β-cyclopentylpropionate. The measurements were carried out at the wavelengths 480, 516 and 552 nm; the calibration curve was prepared with estradiol-3-benzoate.

The procedures of chromatographic separation and gas chromatographic analysis, including the preparation of the derivative, were also performed with the solution (c), the blank of oil only.

RESULTS AND DISCUSSION

Figs. 1 and 2 are photographs of thin-layer chromatograms which show the separation of the estrogens studied, estradiol-17β-cyclopentylpropionate and estradiol-3-benzoate, from the oil. The separation is better for the latter steroid: the conditions used are the result of several experiments carried out with solvent systems already described by us[1].

The results of analysis of solutions with a known quantity of the estrogens studied are presented in Table I. The data obtained show the good reproducibility and accuracy of the procedures studied. As already shown in our previous work[1] concerning the colorimetric analysis, the results obtained reaffirm the applicability of the method described for different derivatives of estrone and estradiol.

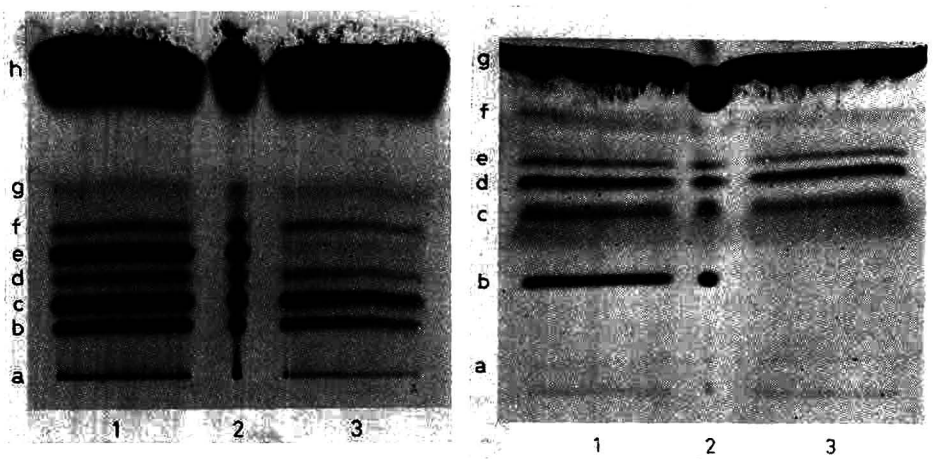

Fig. 1. TLC with HEAA (80:20:1), continuous development for 3 h. (1) Estradiol-17β-cyclopentylpropionate in oil solution; (2) *idem*, reference lane; (3) reference oil blank. (a) Monoglycerides (traces); (b) 1,2-diglycerides; (c) 1,3-diglycerides; (d) and (f) minor components (not identified); (e) estradiol-17β-cyclopentylpropionate; (g) free fatty acids; (h) triglycerides. Detection: 50% H_2SO_4 and heating.

Fig. 2. TLC with HEAA (50:50:1), continuous development for 1.5 h. (1) Estradiol-3-benzoate in oil solution; (2) *idem*, reference lane; (3) reference oil blank. (a) Monoglycerides (traces); (b) estradiol-3-benzoate; (c) 1,2-di-glycerides; (d) 1,3-diglycerides; (e) and (f) minor components (not identified); (g) triglycerides. Detection: 50% H_2SO_4 and heating.

It should be noted here that the procedures of elution and preparation of derivatives described for estradiol-17β-cyclopentylpropionate and estradiol-3-benzoate are applicable for the gas chromatographic analysis of all the derivatives of estradiol.

We think the elution–saponification procedure may be of particular interest as it can be considered as generally applicable to esters of estradiol which can be analyzed as estradiol diTMSE, after saponification; in all cases the same gas chromatographic technique was used.

TABLE I

RECOVERY OBTAINED BY GAS CHROMATOGRAPHIC AND COLORIMETRIC ANALYSIS

The number of determinations is indicated in brackets.

Steroid	*Quantity of steroid used for the analysis (μg)*	*Analytical procedure for separation and gas chromatography*	*Recovery by gas chromatographic method*		*Recovery by colorimetric method (absorbancies are corrected with* ALLEN's *formula)*	
			Mean ± S.D.	*Percentage*	*Mean ± S.D.*	*Percentage*
Estradiol-17β-cyclopentylpropionate in oil solution (2 mg/ml)	40.0	1 (6)	39.7 ± 1.1	99.2 ± 2.7	38.9 ± 1.1	97.2 ± 2.7
Estradiol-3-benzoate in in oil solution (2 mg/ml)	40.0	2 (5)	40.0 ± 1.3	100.0 ± 3.2	40.5 ± 1.0	101.2 ± 2.5

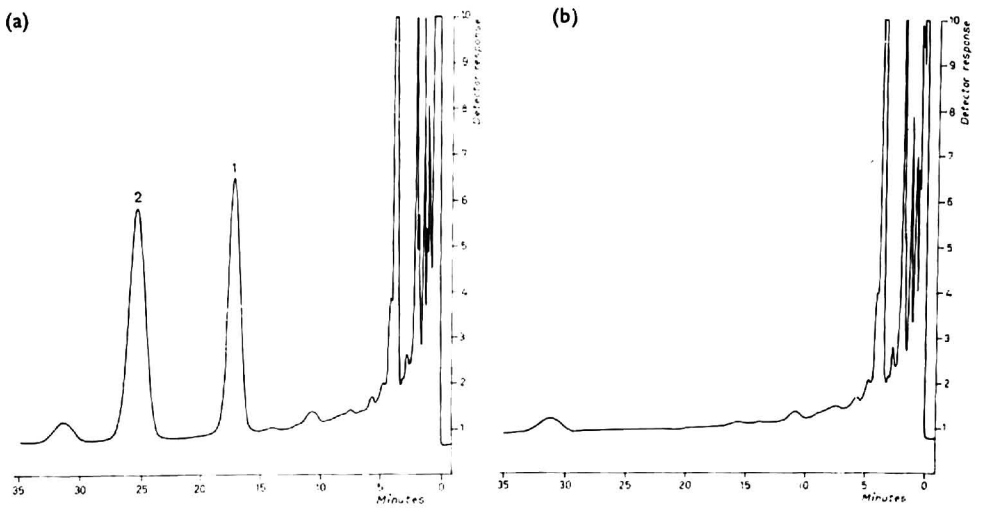

Fig. 3. (a) Gas chromatogram of the eluate of an oil solution of estradiol-17β-cyclopentylpropionate after chromatography, saponification to estradiol, and TMSE preparation. Peak 1: 5α-cholestane. Peak 2: estradiol diTMSE. Column: 2% SE-30 on 80–100 mesh Gas-Chrom Q, 2.20 m length, 190°. (b) Gas chromatogram of the reference oil blank subjected to the same procedure.

The conditions of saponification have been studied in a number of experiments under different conditions, with acids and bases, in water and alcohol media and for different reaction times. The best reaction conditions were those described, giving the highest percentage recovery for the saponified product (also quantitatively controlled by TLC) and assuring the best conditions for the stability of the free estradiol. The saponification procedure and successive gas chromatographic and colorimetric analyses were verified on 40 μg pure estradiol-17β-cyclopentyl propionate*, the following results being obtained: 39.7 $\pm$ 0.8 μg (99.3 $\pm$ 1.9%) by the gas chromatographic method, and 39.9 $\pm$ 1.3 μg (99.9 $\pm$ 3.3%) by the colorimetric one with the correction according to ALLEN[3] (average values of 5 experiments $\pm$ S.D.). Experiments with estradiol gave values of the same order of magnitude.

Our results are comparable with and are, for estrogen alone, better than those obtained by BILLIAR AND EIK-NES[8] after saponification with cholinesterase and sodium carbonate of different steroid acetates, including estradiol. The better results obtained by us with alkali in the presence of silica gel are probably due to the buffering effect of the latter; this is supported by the observations of HORNSTEIN *et al.*[9] on the saponification of lipids under conditions similar to those of our experiments.

The simultaneous elution and saponification procedure can be advantageous not only for the gas chromatographic analysis but also for the colorimetric one: For example, in the case of estradiol-17β-cyclopentylpropionate the direct determination of the estrogen in the chloroform eluate from the silica gel was upset by interfering substances which considerably lowered the recovery and caused its inconstancy. This interference disappeared when performing the analysis with the saponification prod-

* The silica gel blank was obtained from an area, 7.5 $\times$ 2 cm, of a layer developed without a sample.

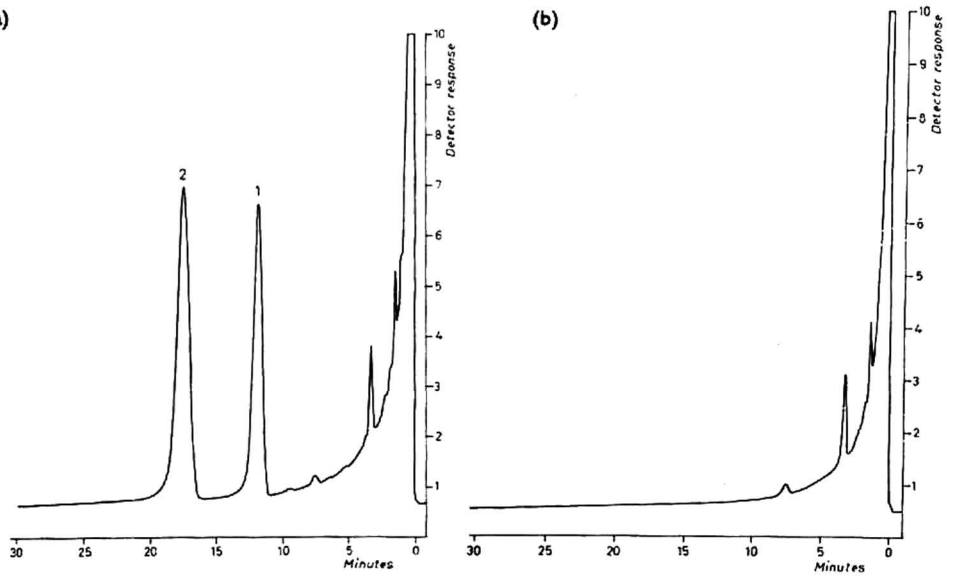

Fig. 4. (a) Gas chromatogram of the eluate of an oil solution of estradiol-3-benzoate after chromatography. Peak 1: cholesteryl propionate. Peak 2: estradiol-3-benzoate-17-TMSE. Column: 2% SE-30 on 80–100 mesh Gas-Chrom Q, 2.20 m length, 235°. (b) Gas chromatogram of the reference oil blank subjected to the same procedure.

uct. In Fig. 1 the complexity of the chromatographic separation is clearly shown. As the concentration of the estrogen is only 0.2% w/v in the oil solution, every component of the oil present with this order of concentration and, with an R_F value near to that of the substance to be separated, can cause interference.

The possibility of choosing convenient techniques of separation, elution and determination also permits one to overcome those difficulties which may be encountered with different substances in different ways.

The second procedure is simpler in its application and allows a more rapid gas chromatographic analysis of the monoesters of estradiol, when they can be eluted without interference from thin-layer chromatograms.

Figs. 3(a) and (b) and 4(a) and (b) show the gas chromatograms of the estrogens studied and the respective oil blanks. They show the good separation of the peaks of the steroids and of the internal standards from some other low peaks which are due to unknown components in the oil.

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A NEW METHOD FOR THE DETERMINATION OF METYRAPONE IN PLASMA AND TISSUES

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SUMMARY

A method is described for the determination of metyrapone and its metabolite(s) after extraction from biological samples. The procedure consists of thin-layer or paper chromatographic separation, the KÖNIG reaction and spectrophotometric determination.

INTRODUCTION

Although metyrapone (Metopirone® CIBA, SU-4885, 2-methyl-1,2-bis(3-pyridyl)-1-propanone) has been widely used for diagnostic purposes for 10 years, very little is known about the metabolic fate of this drug because sensitive, accurate and relatively simple methods for its determination in biological samples are still lacking.

The method described here may fulfill this need and has in fact already been utilized for investigating the metabolism of metyrapone *"in vivo"*[1,2].

The principle is based upon the extraction of metyrapone from biological samples with methylene chloride, separation of metyrapone from its metabolites by paper or thin-layer chromatography and spectrophotometric determination of the product obtained after performing the KÖNIG reaction[3].

EXPERIMENTAL

Biological materials and extraction

Plasma samples (1.5 ml) of metyrapone-treated rats or tissue homogenates (prepared with 10 parts of water) were shaken with 4 vol. of methylene chloride for 4 min and the methylene chloride layer was separated by centrifugation for 10 min at 3000 r.p.m.

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A portion (5 ml) of the methylene chloride layer was removed and evaporated to dryness in a stream of nitrogen at 40°.

Thin-layer chromatography

Kieselgel G (Merck, Darmstadt) layers, 250 μ thick, on glass plates[4] were used after activation at 110° for 30 min. The dried residue of the sample extracts, dissolved in 0.4 ml methylene chloride, or the reference standards (1–20 μg) were applied to the thin-layer plates. The chromatograms were developed in a methylene chloride–ethanol (100:4) system in an unsaturated chamber[5] at 22 ± 0.5° until the front of the solvent had moved about 15 cm.

Paper chromatography

The extracted samples, dissolved in 0.4 ml methylene chloride, were deposited on Whatman No. 1 paper and were equilibrated for 1 h in the tank before adding the mobile phase of a Bush-type solvent system[6]. Petroleum ether–benzene–methanol–water mixtures were used in two different proportions BL_2 (25:25:35:15) or BL_3 (1:1:1:1), respectively, at room temperature.

Detection of spots

This was carried out by iodine vapour (sensitivity is about 3 μg) or by observation in ultraviolet light (at 254 mμ, sensitivity is about 5 μg).

Detection of metyrapone and its metabolite(s) on chromatograms

Semiquantitative estimation. The chromatograms were sprayed with a saturated ethanolic solution of *p*-amino-salicylic acid (PAS) and placed for 15 min in a chamber containing a few crystals of cyanogen bromide (BrCN). The vapour pressure of this substance is high enough to provide a sufficient concentration for the KÖNIG reaction. This reaction is specific for pyridine derivatives with an unsubstituted α-position. The addition of cyanogen bromide is followed by the opening of the pyridine ring with the formation of glutaconic aldehyde, which reacts with the aromatic amine (PAS in this case) forming an intensely coloured imino-derivative of glutaconic aldehyde[7]. By this procedure, metyrapone and its metabolites produce brownish-violet spots. Of the substances 0.5 μg can be easily detected and recorded by a Xerox photocopying machine (Fig. 1).

Quantitative analysis of metyrapone and its reduced derivative. Attempts to elute the coloured spots on thin-layer chromatograms after performing the KÖNIG reaction were unsuccessful. Therefore after the localization of metyrapone and its reduced derivative by iodine vapour or ultraviolet light on thin-layer plates or paper chromatograms, the respective areas were scraped off or cut out and eluted with 3 ml of ethanol. To centrifugated eluates were added: 0.05 ml of 2.5% (w/v) ethanolic solution of *p*-aminosalicylic acid, 0.25 ml of 20% (w/v) ethanolic solution of cyanogen bromide and 0.05 ml of *N* NaOH.

The absorption maximum of the coloured reaction mixture is at 468 mμ and at 472 mμ for metyrapone and its reduced derivative, respectively (Fig. 2).

The colour of the reaction reaches a maximum after around 20 min and remains unchanged for about 10 min (Fig. 3). For serial determinations of metyrapone and

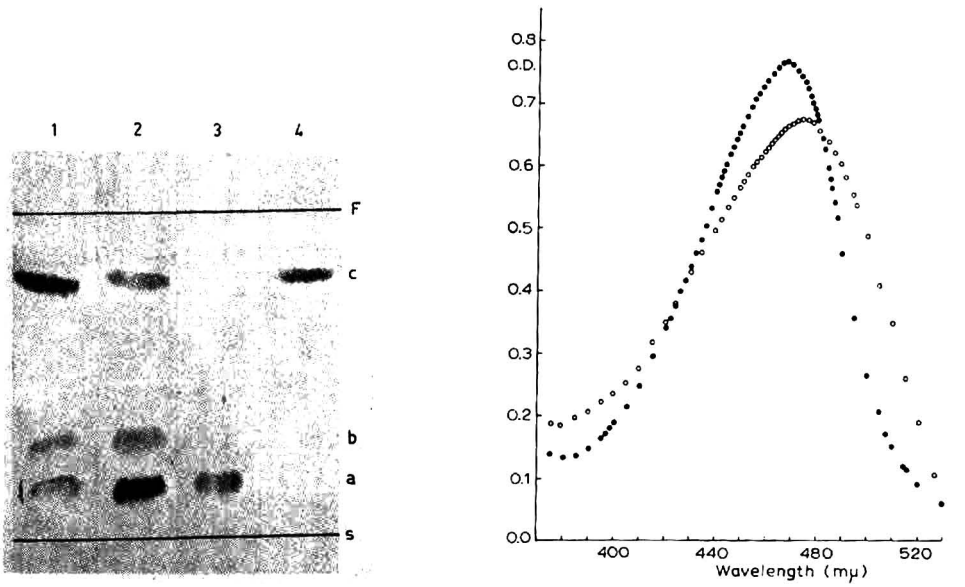

Fig. 1. Thin-layer chromatography carried out according to the method described in the text: (F) front; (a) SU 5236 (2-methyl-1,2-bis(3-pyridyl)-1-propanol); (b) unidentified metabolite; (c) metyrapone; (S) start: 1 and 2; extracts from plasma 20 and 40 min after administration of metyrapone hydrochloride (66 mg/kg i.v.); 3 and 4: pure samples of SU 5236 and metyrapone.

Fig. 2. Optical density of metyrapone (●) or SU 5236 (○) at different wavelengths. Maximum absorption for metyrapone and SU 5236 are respectively 468 and 472 mμ.

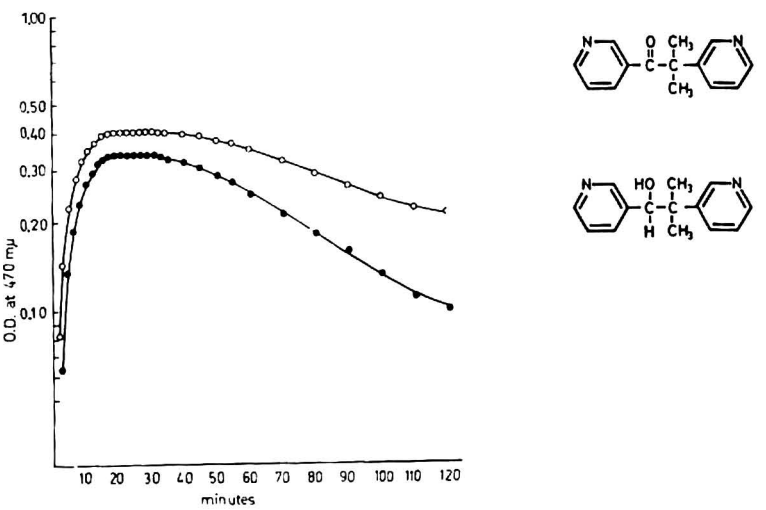

Fig. 3. Development and stability of colour developed after performing KÖNIG reaction on metyrapone (●) or SU 5236 (○). Optical density (O.D.) was measured at 470 mμ.

Fig. 4. Upper formula: 2-methyl-1,2-bis(3-pyridyl)-1-propanone (metyrapone); lower formula: 2-methyl-1,2-bis(3-pyridyl)-1-propanol (SU 5236).

TABLE I

R_F VALUES OF METYRAPONE AND SU 5236 IN DIFFERENT CHROMATOGRAPHIC SYSTEMS

Compound	R_F *in system**		
	1	*2*	*3*
Metyrapone	0.79	0.62	0.81
SU 5236	0.16	0.01	0.05

* 1 = Thin-layer chromatography: methylene chloride–ethanol (100:4 v/v). 2 = Paper chromatography: petroleum ether–benzene–methanol–water (25:25:35:15). 3 = Paper chromatography: petroleum ether–benzene–methanol–water (1:1:1:1). The R_F values were the same for pure substances and for the spots obtained from rat plasma. Detection of the spots was made by the KÖNIG reaction or iodine vapour or U.V.

its metabolite the optical densities were measured at 470 mμ, 25 min after the beginning of the reaction.

By this procedure 1 μg of metyrapone dissolved in water can be determined. The reaction is about 3-fold more sensitive for the reduced metabolite.

The extraction efficiency from rat plasma was 78 $\pm$ 4% (n = 10). The absorption was linear with respect to concentration between 5 and 40 μg for metyrapone and between 2 and 20 μg for the reduced metabolite. The method allows the measurement of a minimum of 5 μg of metyrapone or 2 μg of SU 5236 in 1 ml of rat plasma.

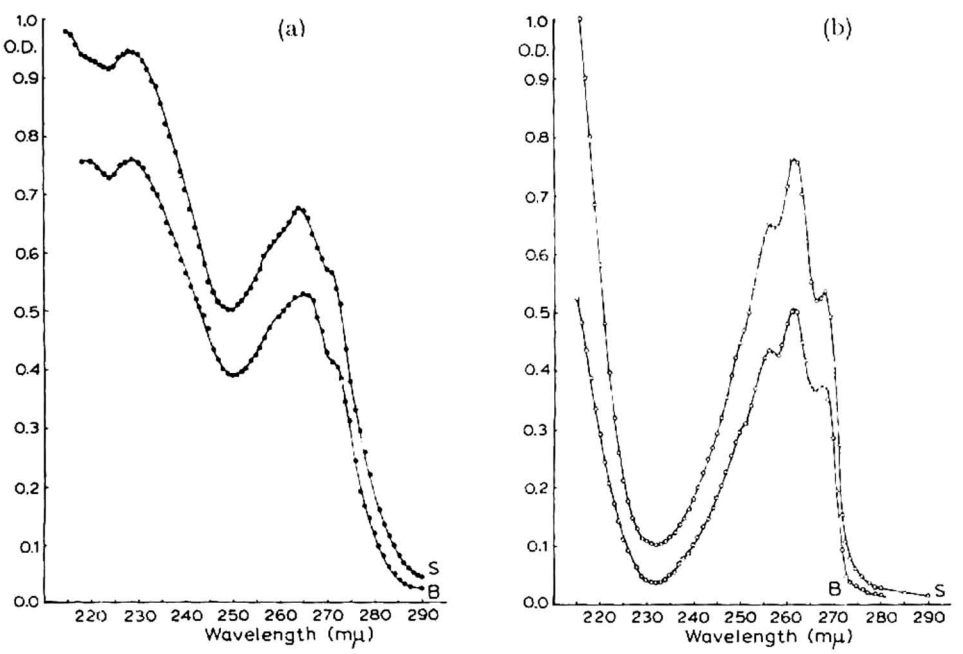

Fig. 5. (a) U.V. absorption spectrum in methanol of metyrapone (S) and of the substance extracted from plasma (B) showing an R_F of 0.79 after thin-layer chromatography. (b) U.V. absorption spectrum in methanol of SU 5236 (S) and of the substance extracted from plasma (B) showing an R_F of 0.16 after thin-layer chromatography.

Identification of the substances extracted from rat plasma and tissues

This was carried out by measuring the R_F values and comparing them with those for pure substances after chromatography in three different solvent systems and by the identification of ultraviolet spectra.

The main metabolite of metyrapone is a reduced derivative (SU-5236: 2-methyl-1,2-bis(3-pyridyl)-1-propanol) found in urine[8] and the incubation fluid of rat adrenal glands and other tissues[9] (Fig. 4).

The R_F values of the substances extracted from rat plasma or tissues and those of the pure materials are the same (Table I).

The ultraviolet absorption spectrum of metyrapone and the spot recovered from rat plasma after administration of metyrapone to rats gave a characteristic triplet, containing maxima at 258, 264 and 270 mμ. SU-5236 and the spot recovered from rat plasma showed another triplet but with the maxima shifted to shorter wavelengths (256, 262 and 267 mμ). The maximum at 229 mμ in the spectrum of SU-4885 was absent in the spectrum of SU-5236 (Fig. 5).

These results show that the substances extracted from rat plasma after the administration of metyrapone are identical with metyrapone and SU-5236, in agreement with the findings of KRAULIS *et al.*[9].

In addition, a spot of a less polar substance giving the KÖNIG reaction was found on the thin-layer plates.

Other spots were also detected sometimes by the KÖNIG reaction which would indicate the presence of trace amounts of similar metabolic products.

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The advice of Prof. A. BIZZI has been very important in the development of this method.

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CHROM. 3933

THE CHROMATOGRAPHIC SEPARATION OF HIGHER FATTY ACID MONOGLYCERIDES ACCORDING TO CHAIN LENGTH AND UNSATURATION*

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SUMMARY

A mixture of monostearin, monopalmitin, monoolein, monolinolein and monolinolenin (5 μg each or more) was obtained by high temperature glycerolysis of vegetable oils and isolated from an equilibrium mixture by liquid–liquid extraction in the system hexane–80% aqueous methanol saturated with boric acid. The individual monoglycerides were then separated by reversed-phase partition chromatography in the system octyl acetate–75% aqueous methanol saturated with $AgNO_3$ and octyl acetate and made visible on the chromatograms with a permanganate solution. The R_2' values of monoglycerides were 2.91, 4.94, 10.42, 12.26, and 13.47, respectively. Preparative separation of individual monoglycerides and gas-chromatographic determination of their fatty acid composition have shown that the main component content of the individual monoglycerides was equal to 96.3–99.5%. The purity of the monoglycerides at all stages of the procedure was checked by adsorption chromatography on alumina. There is no intermolecular isomerization of monoglycerides and no separation of their positional isomers in the reversed-phase system.

INTRODUCTION

The triglyceride molecular species composition of poppy, cotton and linseed oils, as well as oils of other plants has been established earlier in our laboratory[1–4]. As a further development of these studies we intend to investigate the structure (positional species composition) of individual di- and triacid triglycerides of natural oils, *i.e.* to determine the molar ratio of the positional isomers (AAB + BAA):(ABA) in diacid molecules having general formula A_2B, and the ratio of three isomers (ABC + CBA):(ACB + BCA):(CAB + BAC) in triacid molecules of the (ABC) kind.

Until recently it has been impossible to obtain any natural triglyceride in a

* Abbreviations and conventions: A, B, C = any fatty acids, and acyls of these acids in the triglyceride molecule; the sequence of the symbols indicates the position of the acyl in the triglyceride molecule. R_2' = ratio of R_F of monoglyceride to the R_F of butyl hexabromostearate[1]. V_R = retention volume in gas–liquid chromatography. 16:0, 18:0, 18:1, $18:2^{9,12}$, $18:3^{9,12,15}$ = palmitic, stearic, oleic, linoleic, and linolenic acids, respectively.

sufficiently pure individual state, and for this reason its structure was calculated indirectly from the fatty acid composition of total monoglycerides separated by adsorption chromatography of partial glycerides produced by positionally-specific lipase hydrolysis of a natural oil[5–7]. It is evident that in order to investigate directly the structure of an individual triglyceride, it is necessary to have available firstly a method of extraction and purification of the compound from its natural source, and secondly a method of chromatographic separation and identification of individual monoglycerides of definite fatty acid composition, which are produced by enzymic splitting of a triglyceride. The first of these requirements may be considered, at present, as being generally satisfied[1–3], but the chromatographic methods available have not as yet solved the other problem. Silicic acid thin-layer chromatography does not separate monoglycerides according to their fatty acid composition; furthermore, acyl migration caused by adsorption yields unidentified compounds which have the same R_F value as monoglycerides[8,9]. High-temperature gas–liquid chromatography fractionates the volatile trimethylsilyl derivatives of C_8–C_{16}-saturated monoglycerides according to chain length; however, synthesis of these derivatives is somewhat difficult, unsaturated and saturated monoglycerides of the same chain length do not resolve, and the peak of each monoglyceride is split into separate peaks of 1- and 2-isomers making evaluation of the chromatogram very complicated[10,11].

The aim of this investigation was to develop a simple analytical and preparative method for separating monoglycerides of the most common fatty acids occurring in plant tissues, according to chain length and unsaturation. The results obtained indicate that this separation can be achieved by partition chromatography of π-complexes of monoglycerides with silver ions on hydrophobic stationary phases of a new type.

EXPERIMENTAL

Purification of reagents

Methanol, ether, *n*-hexane and *n*-decane were purified according to YURYEV[12]; *n*-octanol-1 and *n*-decanol-1 were distilled before use. Reagent grade glacial acetic acid, acetic anhydride, 96% ethanol, glycerol, acetone, benzene for cryoscopy, $AgNO_3$, H_3BO_3, $KMnO_4$, anhydrous Na_2SO_4, NaOH, NaCl and argon were used without further purification.

Technical grade boric acid was recrystallized, 40 g of H_3BO_3 were dispersed in 100 ml of water and a small amount of charcoal was added; the mixture was heated with frequent stirring until all the H_3BO_3 dissolved, then filtered on a heated funnel and kept overnight at 0°. The H_3BO_3 crystals were washed with iced water on a Buchner funnel and dried to constant weight over concentrated sulphuric acid.

Glycerolysis of linseed and cottonseed oils

A mixture of 10 g of linseed oil obtained by cold pressing, 2.5 g of glycerol and 0.01 g of NaOH as a catalyst was placed in a 100 ml double-necked pear-shaped flask and heated at 180° ± 2° (20 mm Hg) for 4 h with a constant flow of argon. The contents of the flask were cooled to room temperature in the inert atmosphere and transferred with a minimal volume of ether into a 100 ml separatory funnel. The excess of glycerol was removed by 4- or 5-fold extraction with 20% (w/v) aqueous

NaCl; the ether layer was dried with Na_2SO_4, the solvent was distilled off and a 10% (w/v) solution of the glyceride equilibrium mixture in hexane or benzene was prepared. If it was necessary to obtain an equilibrium mixture with a higher content of saturated monoglycerides, a mixture of 9 g of solvent extracted cottonseed oil and 1 g of stearic acid of 96% purity was used instead of linseed oil.

Isolation of monoglycerides

The monoglycerides were extracted five or six times from a hexane solution of the equilibrium mixture with equal volumes of 80% (v/v) aqueous methanol saturated with H_3BO_3. The methanol was distilled off *in vacuo* and the monoglycerides were extracted by 5 × 4 ml of ether, dissolved in benzene to obtain 1% (w/v) solutions, and stored in dry ice to exclude any acyl migration and autooxidation. The purity of the monoglyceride preparation and the completeness of extraction was checked by chromatography on paper impregnated with Al_2O_3 (ref. 2) and also treated with a saturated solution of H_3BO_3 in methanol. The glycerides (100–500 μg) were applied from a micropipette on the start line of a 2.3 × 13 cm paper strip. The separation was carried out as described earlier[2] using 5 ml of ether–decane (4:1 v/v) as the mobile phase.

After the solvent front had reached the upper edge of the chromatogram the paper strip was removed from the chamber, the solvent was removed in a current of warm air, and the glycerides were visualized as brown spots on a light background by immersing the strip for 30–60 sec in a freshly prepared 0.1% (w/v) aqueous solution of $KMnO_4$ (ref. 13). An equilibrium mixture of mono-, di-, and triglycerides has been separated by the same method.

Preparation of stationary phases for reversed-phase chromatography

To synthesize octyl acetate, a mixture of octanol and acetic anhydride (2:3, v/v) was heated to 100° for 48 h and the b.p. 231–232° fraction was separated. In a similar way decyl acetate (b.p. 247–248°) was derived from decanol[14]. The refraction index was measured on an IRF-22 refractometer. The composition of the ester preparations was determined by gas–liquid chromatography.

Chromatographic separation of monoglycerides

Monoglycerides (5 μg or more) from a 1% (w/v) solution were applied as described above on a standard strip[15] of the "Goznak" or "Filtrac N 1" (VEB Spezialpapierfabrik, Niederschlag, G.D.R.) chromatographic paper. The strip was then impregnated with a 5% (v/v) benzene solution of octyl acetate. The monoglycerides were separated for 15–18 h at 20° (ref. 15), using 5 ml of 75% (v/v) aqueous methanol saturated with $AgNO_3$ and octyl acetate as a mobile phase. After evaporation of the methanol the stationary phase was removed in a current of warm air. The strips were then washed of the excess of silver ions and stained with a $KMnO_4$ solution (see above). The R_2' values were determined in relation to trilinolein[16].

Chromatography of monoglycerides on a preparative scale

The sample was applied and separated as described above, but its amount was increased up to 5000–6000 μg, and the time of separation up to 24 h. The monoglyceride spots were cut out from one or several stained chromatograms, cut finely

with scissors and extracted with 1% (v/v) CH_3COOH in ether. Five minutes later the liquid was decanted and the residue was washed several times with ether. Individual monoglycerides were dissolved in benzene and stored in dry ice. The fatty acid composition of the equilibrium mixtures, of the total monoglycerides, and of the individual monoglyceride preparations was determined by gas–liquid chromatography[2].

RESULTS AND DISCUSSION

For a preparative separation of total monoglycerides use was made of an equilibrium mixture of mono-, di-, and triglycerides obtained by glycerolysis of vegetable oils. The concentrations of all components of this system are random[17,18]. Therefore, it was first necessary to determine to what extent the finally adopted parameters of the reaction provide an optimal yield of monoglycerides. As shown in Fig. 1a, our mixture contains approximately equal amounts of mono- and diglycerides

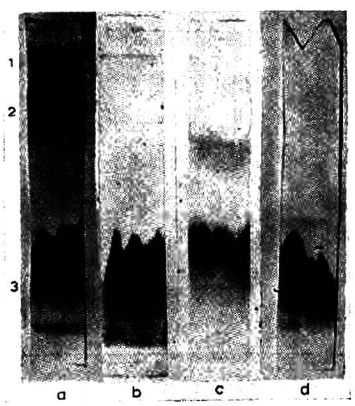

Fig. 1. Adsorption chromatograms of mono-, di-, and triglycerides. For the conditions of separation and development see "EXPERIMENTAL: Isolation of monoglycerides". 1 = Triglycerides; 2 = diglycerides; 3 = monoglycerides. (a) Equilibrium glyceride mixture; (b) monoglycerides isolated by liquid–liquid extraction; (c) the same as (b) + 1% of diglycerides; (d) monoolein eluted from a partition chromatogram.

and a small amount of triglyceride. These results agree with the data of RYBICKA[17] and CHOUDHURY[18] according to whom the quantities of the respective products of glycerolysis of linseed oil amounted to 44–45, 44–51, and 5–10%. It is clear that our equilibrium system contains enough of the monoglycerides for their preparative isolation.

The total monoglycerides were isolated by liquid–liquid extraction according to the modified technique of GALANOS AND KAPOULAS[19] originally used by these authors to separate polar lipids in the two-phase system petroleum ether–87% ethanol. Boric acid saturated aqueous methanol served as the lower phase for the separation of the monoglycerides, the borate anions being capable of forming hydrophilic complexes with free hydroxyl groups of monoglyceride[20]. The method suggested can effect almost complete extraction of the monoglycerides from a non-polar phase.

TABLE I

FATTY ACID COMPOSITION OF EQUILIBRIUM MIXTURES AND ISOLATED MONOGLYCERIDES (moles %)

Original oil	*Products of glycerolysis*	*16:0*	*18:0*	*18:1*[9]	*18:2*[9,12]	*18:3*[9,12,15]	*Average lipophily of fatty acids*[21]
Linseed oil	Equilibrium mixture	6.8	4.6	17.6	10.7	60.2	13.4
	Monoglycerides	4.8	2.5	13.8	10.4	68.5	13.1
Cottonseed oil + stearic acid (9:1)	Equilibrium mixture	27.4	9.2	15.5	47.8	—	15.2
	Monoglycerides	13.0	8.6	16.9	61.5	—	14.9

The diglycerides do not pass into the aqueous phase: their visually estimated content in the final monoglyceride preparation amounted to less than 1% (Fig. 1b, c). The extractability of various monoglycerides may be different depending on chain length and unsaturation. A parallel determination of the fatty acid composition (Table I) has shown that, compared with the original mixture, the monoglycerides exhibit a somewhat lower lipophily and, consequently, a greater polarity of their acyl radicals[21]. Nevertheless, the concentrations of fatty acids found are quite sufficient for the separation of each individual monoglyceride. It should be emphasized that the extraction method has certain advantages over vacuum distillation[22,23] which yields no monoglycerides with a purity better than 50–60% and leads to a considerable acyl migration; it also compares favourably with the dialysis through a rubber membrane[24], which is very time-consuming.

Our preliminary attempts were to separate monoglycerides with regard to their fatty acid composition by reversed-phase partition chromatography in the system *n*-dodecane–silver nitrate saturated 80–95% methanol[16], because this method had been used successfully in the original form or in a somewhat modified form[25,26] for the fractionation of triglycerides and methyl esters of saturated and unsaturated fatty acids according to chain length and number of double bonds. However, because of the poor solubility of the coordination complexes of monoglycerides with silver ions in *n*-dodecane, their separation in this system proved unsatisfactory. The use of the aromatic hydrocarbon tetralin as a lipophilic phase did not improve the results. We assumed that the affinity of aliphatic stationary phase to more polar substances will increase if some hydrophilic functional group is introduced into the hydrocarbon chain. It finally turned out that if the *n*-dodecane used in the previous system was replaced by *n*-octyl or *n*-decyl aliphatic alcohols the differences in the chromatographic mobility of individual unsaturated monoglycerides considerably increased. However, the time of their separation also increased, and the separation of the monoglycerides of palmitic and stearic acids in the system *n*-octanol–silver nitrate saturated 50–60% methanol was extremely slow. Their affinity to the mobile phase could not be increased by reducing the water content in the phase because of the illimitable solubility of *n*-octanol in such solutions. Therefore, it became necessary to find a stationary phase which

TABLE II

CHARACTERISTICS OF STATIONARY PHASES FOR CHROMATOGRAPHIC SEPARATION OF MONOGLYCERIDES*

Stationary phases	*Boiling point (°C, 760 mm Hg)*		*Refraction index* n_D^{20}		V_R, *ml of carrier gas***	*Ratio between* V_R *values*	
	Found	*Literature*	*Found*	*Literature*		*Found*	*Literature*
n-Octanol-1	195	195[27]	1.4303	1.4304[28]	492	0.55	0.59[29]
n-Decanol-1	231	231[27]	1.4380	1.4368[27]	902		
n-Octyl acetate	207	210[30]	1.4207	1.4204[31]	456	0.59	0.56[33]
n-Decyl acetate	242	244[32]	1.4279	1.4273[32]	770		

* The superior figures are literature references.

** Operating conditions are similar to those described earlier[2], but column temperature is 125°, and the carrier gas flow rate 15 ml/min.

Fig. 2. Partition chromatogram of monoglycerides. For the conditions of separation and development see "EXPERIMENTAL: Chromatographic separation of monoglycerides". Sample weight 1000 μg. 1 = Monolinolenin; 2 = monolinolein; 3 = monoolein; 4 = monopalmitin; 5 = monostearin.

would be intermediate, by the degree of polarity, between a hydrocarbon and an aliphatic alcohol. It was suggested that the required value of polarity could be obtained by introducing an ester group into the aliphatic chain. To check this hypothesis, the synthesis of acetates of the two aliphatic alcohols was carried out. Some properties of the resultant esters as well as of the original alcohols are shown in Table II. It can be seen that physico-chemical constants found for the compounds investigated are close to those published elsewhere.

As shown in Fig. 2, the system *n*-octyl acetate 75% aqueous methanol saturated with $AgNO_3$ and octyl acetate allows a clear separation of individual monoglycerides. It should be emphasized that, disregarding earlier investigations[34,35] in which the paper was impregnated with triglycerides of olive oil to separate free fatty acids, the aliphatic stationary phases containing an ester group in the chain are now being used for the first time in the liquid partition chromatography of lipids. Up to now, hydrocarbon phases have been used almost exclusively in reversed-phase chromatography while stationary phases containing ester groups were only used in gas-liquid chromatography of lipids. It could be that acetates of higher fatty alcohols will find application in the partition chromatography of many classes of lipids.

Individual monoglycerides were identified by their relative chromatographic mobility, by the reaction of permanganate with their double bonds and by the fatty acid composition of their preparations. It is evident from Fig. 2 and Table III that the mixture of monoglycerides is separated into five zones having R_2' 2.91, 4.94, 10.42, 12.26, and 13.47. The three upper zones which are stained brown by a permanganate solution are apparently monoglycerides of unsaturated acids. The two lower spots do not give a colour reaction, but they are easily discernible in transmitted light on a moist chromatogram as dark areas against a more transparent background; in reflected light these zones are visible as white areas on the light-pink surface of the paper. It is suggested that the lower zones are produced by saturated monoglycerides. Determination of the fatty acid composition of the monoglycerides after their elution (Table III) allowed the final identification of the above zones as monostearin, monopalmitin, monoolein, monolinolein and monolinolenin, respectively. When separated in the reversed-phase system containing silver ions, individual monoglycerides are arranged on the chromatogram in the same order as the methyl esters

TABLE III

RELATIVE CHROMATOGRAPHIC MOBILITY, FATTY ACID COMPOSITION AND IDENTIFICATION OF INDIVIDUAL MONOGLYCERIDES

Number of chromatographic zone (Fig. 2)	R_2'	*Fatty acid composition of individual zones (moles %)*					*Identification*
		16:0	*18:0*	*18:1*[9]	*18:2*[9,12]	*18:3*[9,12,15]	
5	2.91	3.7	96.3				Monostearin
4	4.94	99.1		0.9			Monopalmitin
3	10.42	2.8	0.4	96.8	—		Monoolein
2	12.26	1.7	—	—	98.3		Monolinolein
1	13.47	0.5		—		99.5	Monolinolenin

of their respective fatty acids[16,26], *i.e.* in the order of the increasing polarity of the monoglycerides and their coordination complexes.

It is well known that under drastic conditions of separation and preparative isolation monoglycerides may undergo intermolecular isomerization with the appearance of di- and triglycerides, and intramolecular isomerization which causes the conversion of 2-monoglycerides into 1(3)-isomers[9,23]. As shown arbitrarily for monoolein (Fig. 1d), the eluted monoglycerides do not contain di- and triglyceride impurities, which points to the absence of intermolecular acyl migration. No definite answer can as yet be given to the question as to whether the partition chromatography and elution of the monoglycerides are or are not accompanied by their intramolecular isomerization, since monoglyceride positional isomers are not separated in our adsorption and reversed-phase chromatographic system.

Thus, it should be concluded, that the reversed-phase partition chromatographic system containing silver ions in the mobile phase and higher fatty alcohol acetates as a stationary phase permits an efficient analytical separation and identification of individual monoglycerides produced by lipase hydrolysis of natural triglycerides. The mild conditions of the liquid–liquid extraction and of the partition chromatographic procedure, which exclude the use of adsorption, higher temperatures and extreme pH's, make it possible to isolate individual fatty acid monoglycerides free from isomerization on a preparative scale.

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CHROM. 3943

INVESTIGATIONS ON THE RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND CHROMATOGRAPHIC PARAMETERS

I. TWO HOMOLOGOUS SERIES OF PYRIDINE DERIVATIVES SUITABLE AS REFERENCE COMPOUNDS IN LIQUID–LIQUID PARTITION CHROMATOGRAPHY

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SUMMARY

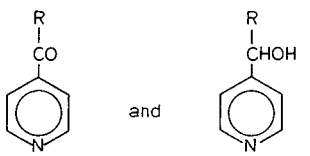
R_M values of several 4-pyridyl alkyl ketones and 4-pyridyl alkyl alcohols determined for a number of aqueous and non-aqueous liquid–liquid partition chromatographic systems, were found to be linearly dependent on the number of carbon atoms in the alkyl chain. The R_M values of the ketones or alcohols varied in the range of optimal accuracy of chromatographic data; the compounds are thus proposed as reference solutes for liquid–liquid partition chromatography, using a method analogous to that of the KOVATS' indices employed in gas–liquid partition chromatography.

INTRODUCTION

In recent years, the interpretation of structural effects in chromatography has developed rapidly, mostly due to the R_M concept; work in this field up to 1965 has been summed up in an excellent review by BUSH[1]; shorter reviews have been written by GREEN AND MARCINKIEWICZ[2], GREEN AND MCHALE[3], LEDERER[4,5] and PROCHAZKA[6]. In liquid–liquid partition chromatography, oxygen-containing organic compounds have been investigated mostly, whereas nitrogen compounds have been avoided, presumably in view of the complicating effect of ionization equilibria on chromatographic behaviour (see, for instance, PARDEE[7] and MOORE AND BAKER[8] for chromatography of peptides and MACEK AND VEJDĚLEK[9] for alkaloids). Because a high proportion of drugs and substances of biochemical importance are organic nitrogen compounds, investigations on the chromatographic behaviour of organic nitrogen bases have been started in this laboratory; the investigations are related to our earlier work on some theoretical problems of liquid–liquid partition chromatography (see, for instance, refs. 10–12). In addition to the theoretical significance of this problem, it was worth considering that among the parameters determining the action of drugs

and their migration kinetics in the body, their solubility and basicity play an important part[13] as they do in liquid–liquid partition chromatography. In other words, if the body is regarded as an extremely complex chromatographic system, in which the blood plays the role of the developing solvent, a certain parallelism can be expected between the behaviour of drugs and their chromatographic parameters in common "simple" partition systems.

In the course of the investigations two homologous series of pyridine derivatives have been obtained



where R is an alkyl of 1–5 carbon atoms. It was found that the compounds are suitable as references for liquid–liquid partition chromatography, since in numerous solvent systems the elongation of the alkyl chain entails a variation of the R_M values in the range of optimal accuracy (−0.5 to +1.0, which corresponds to R_F values 0.75 and 0.1, respectively). This is due to the fact that the first members of the two series, the methyl derivatives, contain two hydrophilic centers in relatively small molecules: the heterocyclic nitrogen and the keto or hydroxyl group. Further advantages of the compounds are that they can be easily prepared, are stable and can be detected easily with Dragendorff's reagent. In the numerous systems investigated (see below) the R_M value was found to be linearly dependent on the number of carbon atoms in the alkyl chain; the two homologous series can thus serve as reference compounds for the determination of the chromatographic parameters of other substances in a manner analogous to the "KOVATS' indices" in gas–liquid partition chromatography[14,15], where *n*-alkanes are used as the reference series and the retention times are expressed by the (fractional) number of carbon atoms of a hypothetical alkane emerging from the column simultaneously with the solute. KOVATS' indices are now extensively employed in gas chromatography since they eliminate to a large extent the irreproducibility of conditions of gas chromatographic analysis. The difficulties in improving the reproducibility of liquid chromatography were extensively discussed at the 3rd Liblice Symposium (1967)[16]; in adsorption chromatography on alumina and silica gel the situation has been improved as a result of introduction of polycyclic aromatic hydrocarbons as reference solutes by SNYDER[17], but the problem seems to remain an open question in liquid–liquid partition chromatography. However, the development of automated equipment for the latter technique provides a stimulus for attempts to solve this problem.

EXPERIMENTAL

The first three ketones were obtained by Claisen synthesis[18] from ethyl isonicotinate and ethyl esters of acetic, propionic and butyric acid, respectively. The remaining two ketones, *i.e.*, pyridyl-butyl ketone and pyridyl-amyl ketone were obtained by reaction of sodium salt of ethyl isonicotinylacetate with propyl bromide

and butyl bromide, respectively[19]. The respective pyridyl alcohols were prepared by reduction of the ketones with sodium borohydride[20].

The compounds were analysed by partition chromatography. Whatman No. 4 paper was cut into strips 7 × 23 cm at right angles to the machine direction and impregnated with water or MacIlvaine's buffer solution (0.2 *M* Na_2HPO_4 + 0.1 *M* citric acid), blotted between two sheets of filter paper and dried in air until the moisture content dropped to 0.5 g of aqueous phase per 1.0 g of dry paper and then transferred immediately to chromatographic tanks, 33 × 5 × 7 cm. The developing solvents possessed various H-bonding properties, thus, cyclohexane, heptane, benzene and carbon tetrachloride belonged to class N (ref. 21), chloroform to class A, di-*n*-butyl ether to class B. The chromatograms were developed to a distance of 16 cm and the spots detected by immersion in a modified Dragendorff's reagent (0.2 g of bismuth subnitrate dissolved in 1 ml of conc. HCl added to 100 ml of 3 w/v % KI). In the second series of experiments less volatile liquids were used as the stationary phase. Whatman No. 4 paper strips were immersed twice, with a 5 min interval, in *ca.* 20 v/v % acetone solutions of formamide, dimethylformamide or ethylene glycol, the excess solution being immediately blotted off between two sheets of filter paper so that the degree of impregnation amounted to 0.5 g of the nonvolatile liquid per 1 g of dry paper.

The experiments were carried out at 22 ± 1°.

The results presented in Figs. 1–8 are averages from at least three experiments.

RESULTS AND DISCUSSION

Aqueous systems

In Fig. 1 the R_F and R_M values of the solutes are plotted against the number of carbon atoms in the alkyl chain n_C, for systems in which pure water was used as the stationary phase. Since R_M values calculated from R_F coefficients beyond the range 0.1 to 0.7 are less accurate, the R_M *vs.* n_C relationships beyond the range +1 to −0.5 are represented by dashed lines. The more commonly employed definition of R_M proposed by BATE-SMITH AND WESTALL is used ($R_M = \log (1 - R_F)/R_F$); however, the R_M axis is directed towards negative (see Fig. 1), as if the REICHL definition were used, so that the R_M and R_F values both increase.

It can be seen from Fig. 1a that the homologous series of alcohols give for aqueous systems regular, linear relationships between the R_M value and number of carbon atoms in the alkyl chain, which provides a further proof of the constancy of the ΔR_M (CH_2) increment for a homologous series. The increment for a single methylene group equals *ca.* −0.60 when cyclohexane, benzene and di-*n*-butyl ether are used as developing solvents which is in excellent agreement with PIEROTTI's data (see Fig. 1 in ref. 1). For the system chloroform–water the R_M *vs.* n_C line is somewhat less steep, the ΔR_M (CH_2) value amounting to *ca.* −0.50.

For strong and moderate extractants (chloroform, benzene), the variation of R_M values with the length of the alkyl chain occurs in a suitable range of R_M values but with nonpolar solvents (cyclohexane) measurable R_M values are only obtained for the higher members of the series. Thus, in order to avoid the extension of the series, the respective 4-pyridylketones are more suitable as reference compounds for nonpolar extractants (see Fig. 1b). In this case the value of ΔR_M (CH_2) is again *ca.* −0.60.

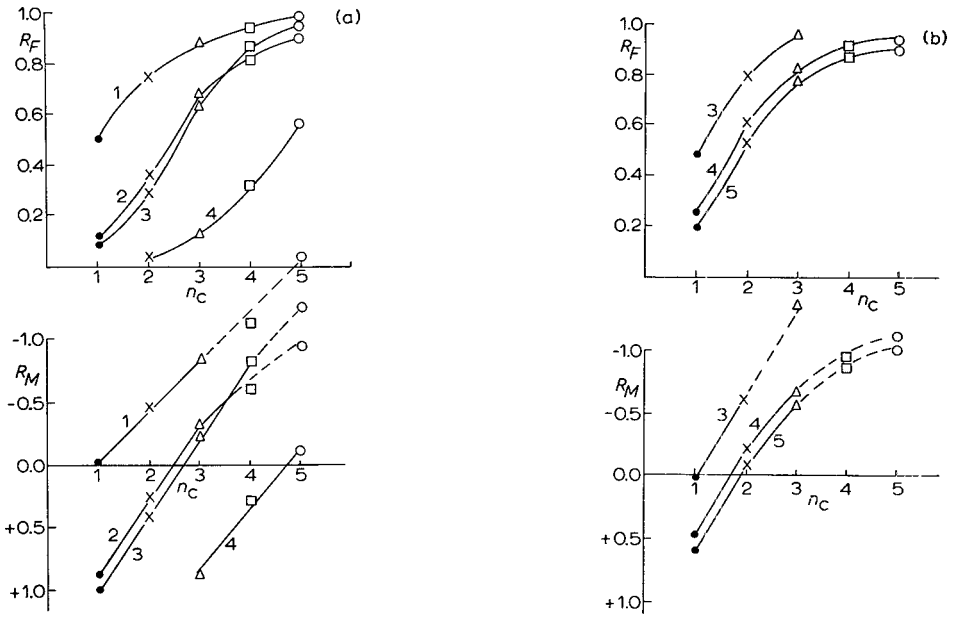


Fig. 1. R_F *vs.* n_C and R_M *vs.* n_C relationships of 4-pyridylalkyl alcohols (a) and the corresponding ketones (b). Stationary phase: water. Mobile phase: chloroform (1), benzene (2), di-*n*-butyl ether (3) cyclohexane (4) or heptane (5).

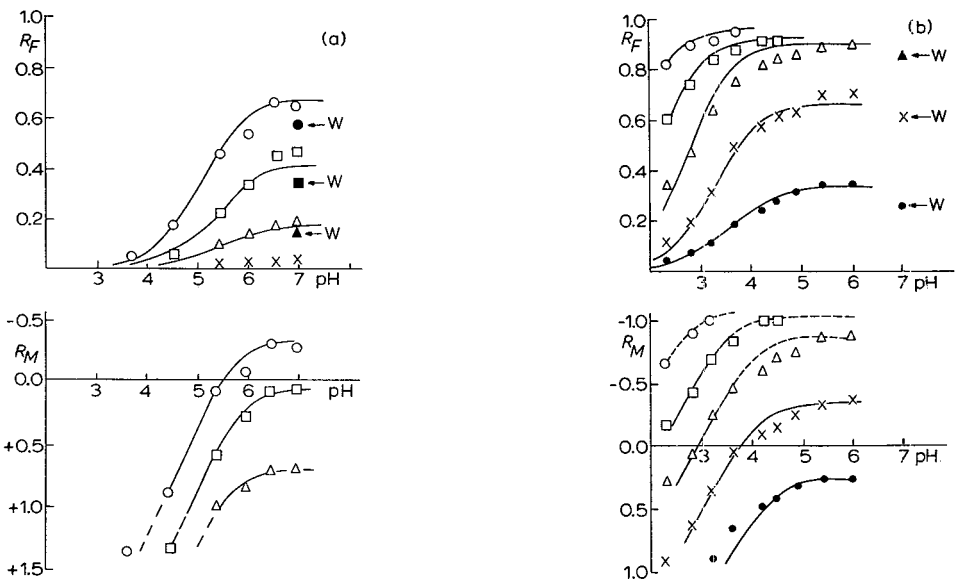


Fig. 2. R_F *vs.* pH and R_M *vs.* pH relationships of homologous 4-pyridylalkyl alcohols (a) and ketones (b). The lines in upper Fig. 2a are theoretical R_F *vs.* pH lines calculated for $pK_A = 5.5$ and $kr = 0.2$, 0.7 and 2.0, respectively. The three lower curves in upper Fig. 2b are theoretical curves for $pK_A = 3.8$ and $kr = 0.5$, 2.0 and 10.0, respectively. W represents R_F values obtained for the system cyclohexane–pure water. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

The points of the butyl and amyl derivatives deviate from the straight line; however, they fall beyond the range of optimal accuracy and this is presumably the cause of this deviation. The series of pyridyl ketones is thus an excellent auxiliary set of reference compounds, the transformation of the =CHOH group into =CO group giving rise to a change of R_M of *ca.* −1.6 units (calculated for the system cyclohexane–water, Fig. 1a, b); which corresponds to *ca.* 3 methylene groups. Fig. 1a visualizes very clearly how the peculiar sigmoidal shape of the R_F *vs.* R_M relationship (see ref. 10, Fig. 4) is responsible for the S-shaped R_F *vs.* n_C relationship (or parts of the letter S: concave for higher values of R_F and convex for lower values).

In the next series of experiments, R_F *vs.* pH relationships were determined for both series of pyridine derivatives using cyclohexane as the developing solvent. For alcohols, low sigmoidal curves were obtained (Fig. 2) which for increasing pH exceed the values obtained with the system cyclohexane–water; this increase of R_F is presumably due to the salting-out effect of the increasing concentration of Na_2HPO_4. The position of the curves seems to indicate[22] that the pK_A value of the pyridyl alcohols is *ca.* 5.5. To obtain measurable R_F values in lower ranges of pH, it would be necessary to increase the alkyl chain by several methylene units. On the other hand, the pyridyl ketones show higher R_F values (Fig. 2b) and are suitable for the calculation of retention indices in the range of pH 2.0 to 4.0 in which the R_M values are linearly dependent on pH. The pK_A values of the ketones estimated from the R_F *vs.* pH curves are somewhat lower than in the case of alcohols and amount to *ca.* 3.7. In addition, in this case the R_F *vs.* pH lines at suppressed ionization (pH >5)

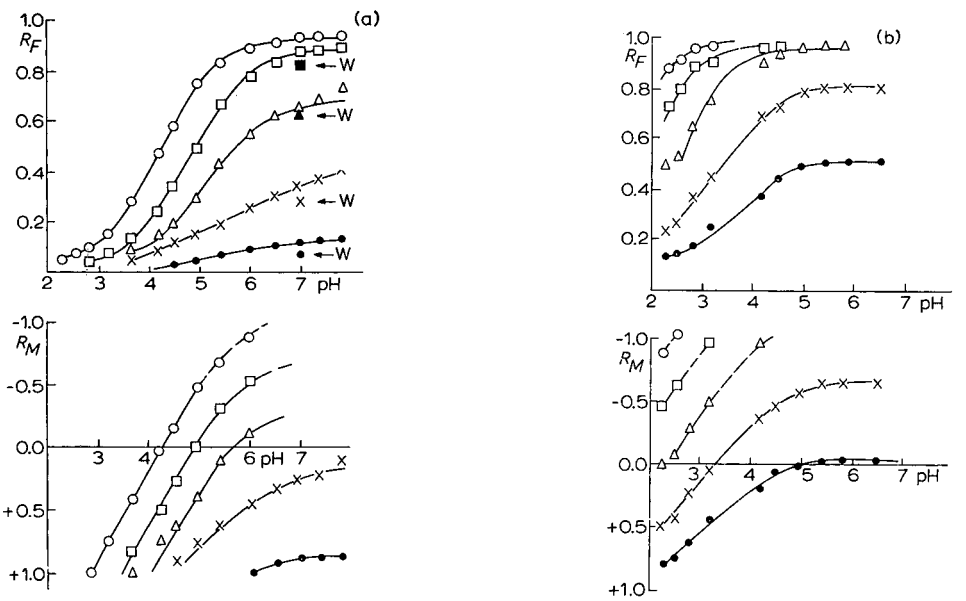


Fig. 3. R_F *vs.* pH and R_M *vs.* pH relationships for the two homologous series chromatographed in the system di-*n*-butyl ether–buffer solution. W represents R_F values obtained for the system cyclohexane–pure water. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

are not horizontal but have positive slopes which indicates that in the theoretical equation[10]:

$$R_F = \frac{kr}{kr + 1 + 10^{pK_A - pH}}$$

also the partition coefficient (k) of unionized base (and perhaps its pK_A as well) is a function of the concentration of Na_2HPO_4 in the buffer phase (salting-out effect). Linear, parallel and regularly spaced R_M *vs.* pH relationships are obtained for the three higher ketones in the range of pH 2.0 to 4.0. Since the carbonyl group shields the pyridine ring from the inductive effect of the alkyl group, the pK_A values of the members of the series are probably similar so that Fig. 2b illustrates a family of R_F

Fig. 4.

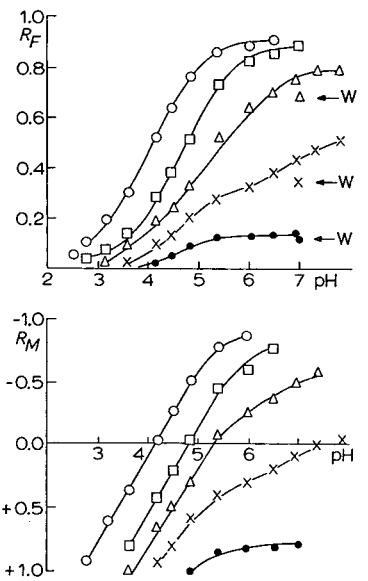


Fig. 5

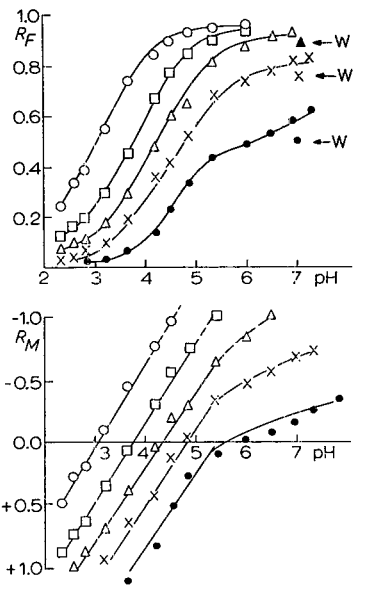


Fig. 4. R_F *vs.* pH and R_M *vs.* pH relationships for the series of alcohols chromatographed in the system benzene–buffer solution. W represents R_F values obtained for the system cyclohexane–pure water. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

Fig. 5. As in the preceding figure; mobile phase: chloroform.

vs. pH curves for various values of the partition coefficient k and a constant value of pK_A (see ref. 10, Fig. 22 and Fig. 19). Analogous plots for di-*n*-butyl ether as the developing solvent are presented in Fig. 3; the curves are higher due to the better extraction of the compounds by butyl ether. At suppressed ionization the ΔR_M (CHOH → CO) is lower as in the case of cyclohexane and amounts to *ca.* −1.0; this is probably due to the specific interaction of the hydroxyl group with the ether oxygen of the organic solvent.

For the benzene system, the R_F *vs.* pH curves are similar to those obtained for butyl ether (Fig. 4); for chloroform the curves are still higher so that accurate R_M values can only be obtained for the series of alcohols (Fig. 5).

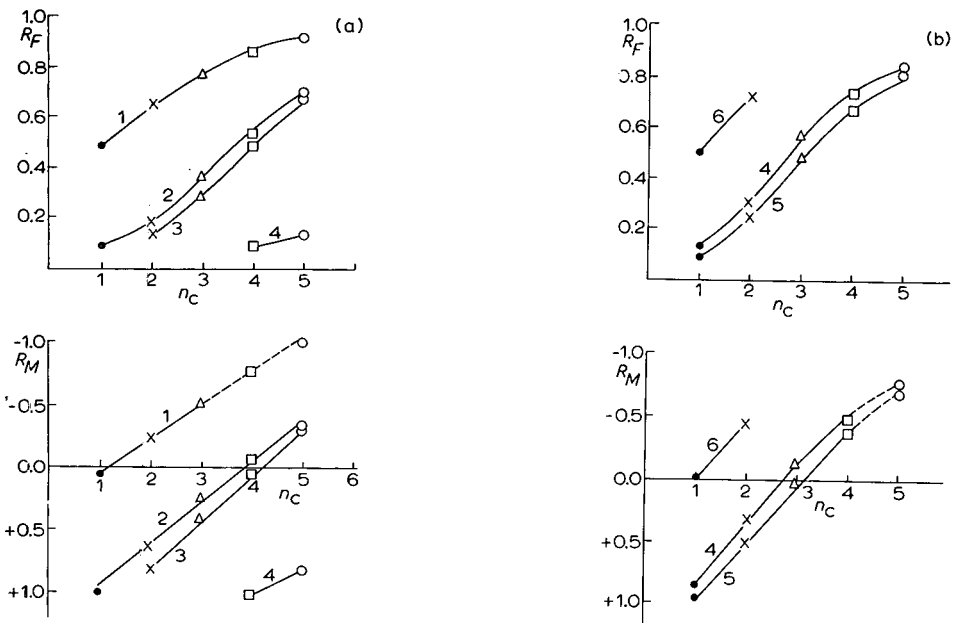


Fig. 6. R_F and R_M values as function of the number of carbon atoms in the alkyl group of the homologous alcohols (a) and ketones (b). Stationary phase: formamide. Mobile phase: chloroform (1), benzene (2), di-*n*-butyl ether (3), cyclohexane (4), heptane (5) and carbon tetrachloride (6). The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

Non-aqueous systems

Results obtained for formamide-impregnated paper are presented in Fig. 6. It can be seen that for strong and moderate extractants, R_M values are obtained for the alcohols in the suitable range (Fig. 6a); for weak solvents, the series of alcohols has to be extended by several additional methylene groups, or substituted by the ketones (Fig. 6b). The R_M values vary linearly in the range of optimal accuracy; the absolute value of ΔR_M (CH_2) is lower than in the case of aqueous systems and amounts to −0.45 to −0.50 for the nonpolar solvents (ketones, Fig. 6b) and −0.40 to −0.30 for mobile phases of moderate or strong solvent strength (alcohols, Fig. 6a).

Similar relationships were obtained employing ethylene glycol as the stationary phase (Fig. 7). The ΔR_M (CH_2) value is still lower and amounts to −0.30 R_M units.

When dimethylformamide (DMF) was used as the stationary phase, the R_F values of the alcohols were too low; therefore, R_F values were determined by using mixtures of cyclohexane and benzene as the mobile phase, (not exceeding 50 v/v % benzene in view of the miscibility of dimethylformamide with the nonpolar phase at higher concentrations of benzene[23]). Linear R_M *vs.* volume composition relationships were obtained in view of the absence of stronger interactions in the less-polar phase[10]. The ΔR_M (CH_2) value is *ca.* −0.2 in the series of alcohols and *ca.* −0.25 in the case of ketones. For the system 40% benzene + 60% cyclohexane/DMF, for which suitable R_F values are obtained for both ketones and alcohols, the difference in the R_M values of the methyl ketone and the methyl alcohol is *ca.* −0.60 R_M units and for the corresponding amyl derivatives *ca.* −0.7 units.

Fig. 7.

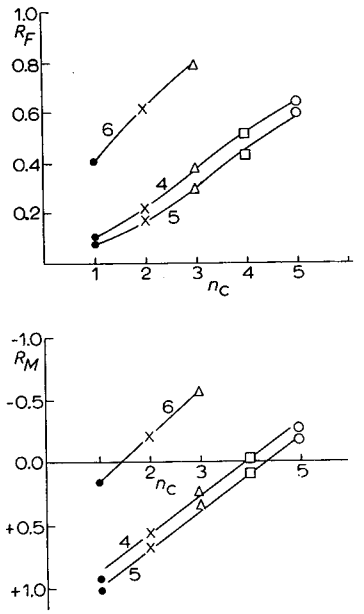


Fig. 8

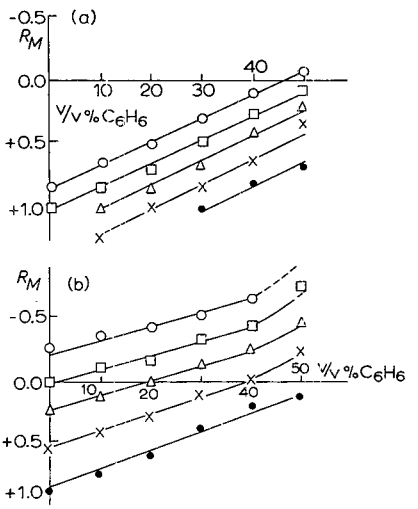


Fig. 7. R_F *vs.* n_C and R_M *vs.* n_C relationships of the ketone series in systems of the type: nonpolar solvent–ethylene glycol. Mobile phases denoted as in Fig. 6. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

Fig. 8. R_M values of the five alcohols (a) and ketones (b) plotted as function of v/v % benzene in the mobile phase, composed of cyclohexane and benzene. Stationary phase: dimethylformamide. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

CONCLUSIONS

The experiments demonstrate linear relationships between the R_M values of 4-pyridyl alkyl ketones and 4-pyridyl alkyl alcohols and the number of carbon atoms in the alkyl chain; deviations from linearity were observed only beyond the range of optimal accuracy (for $R_F > 0.70$) and thus were presumably due to gradient effects.

Suitable ranges of R_F values were obtained for weakly polar solvents of various solvent power and for water, formamide, dimethylformamide and ethylene glycol as the polar phase, which qualifies the homologous series investigated as reference solutes for the determination of chromatographic parameters of other solutes by the KOVATS' retention index method.

The change of R_M due to the reduction of =CO to a =CHOH group amounts to *ca.* -1.6 R_M units for cyclohexane–water and cyclohexane–formamide systems. This change is due to the stronger solvation of the hydroxyl group (and presumably to stronger solvation of the heterocyclic nitrogen in pyridyl alkyl alcohols in view of their higher basicity).

The use of retention indices eliminates or reduces a number of variables which cause irreproducibility of chromatographic data obtained in various laboratories. One such factors in impregnation methods is the variation of the degree of impregnation. Since the R_M values of various solutes vary linearly and are parallel when plotted

against the impregnation degree on a logarithmic scale[25], it can be expected that the use of a homologous series as a reference system will largely eliminate (or at least reduce) this effect, because a change of the ratio of volumes of the two phases should shift all R_M coefficients by a constant value.

On the other hand, when irreproducibility is caused by variation of composition of the solvent (or the pH of the aqueous phase[24]), the discrepancies can be expected to be reduced if the dependence of R_M upon the composition (or pH) is approximately parallel for the solutes investigated and for the reference solutes; thus, in systems with buffered aqueous phases the pyridyl alcohols will be useful in the range of pH 2 to 5 in which linear R_M *vs.* pH relationships are obtained. Other monoprotic bases strongly ionized within this range (*i.e.*, having $pK_A > 5$) will most probably give linear R_M *vs.* pH relationships parallel to those of the reference compounds. If, however, the relationships of the reference substances are not parallel to those of the solutes which are being investigated, then it is at least possible to check on the basis of the R_F values of the reference solutes whether the conditions of the chromatographic experiments have been properly reproduced.

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CHROM. 3950

CHROMATOGRAPHIC AND ELECTROPHORETIC STUDY OF ARYLOXYALKYL AMMONIUM COMPOUNDS

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SUMMARY

The chromatographic and electrophoretic behaviour of aryloxyalkyl ammonium compounds, such as 1-alkylpiperidinium bromides, pyridinium bromides, benzyldimethylammonium bromides and trimethylammonium bromides, were studied. The group constants for the aromatic, alkyl and basic parts of the molecules of the compounds studied were determined in the chromatographic system formamide–1,1′,2,2′-tetrachlorethane. In all cases there was good agreement between the calculated and the experimental R_F values. The relationship between the structure of the aryloxyalkyl ammonium compounds and their chromatographic and electrophoretic properties is discussed.

INTRODUCTION

Organic quaternary nitrogenous compounds represent a pharmaceutically interesting group of substances with antimicrobial, fungicidal, local anaesthetic, sympatholytic and other effects. Their surface-active and solubilizing properties[1] are also of importance.

Soon after 1943 when RAWLINS *et al.*[2] prepared a series of these compounds with germicidal activity, increasing interest in them became apparent[3–11]. The solubilizing properties of ammonium salts of the $(Ar\text{-}O(CH_2)_n\text{–}\overset{|}{\underset{|}{N}}\text{–})^+Br^-$ type depend to a great extent on the structure of the solubilizer and on the type of solubilized substance[12]. Basic information concerning the relationship between structure and chemical behaviour of organic ammonium salts can be obtained by partition paper chromatography and electrophoresis. In addition to this, both these methods can be used for a quick check on identity and purity during their preparation.

MATERIALS AND METHODS

Chemicals

The quaternary nitrogenous compounds studied, listed in Table I, were prepared by synthesis according to previously described procedures[12]. The chemicals

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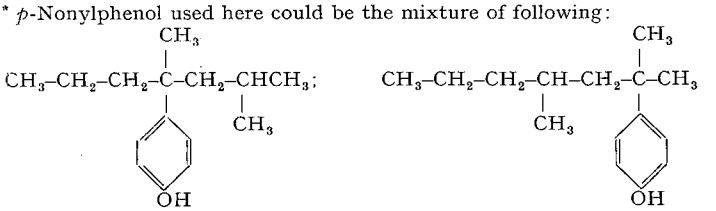
TABLE I

ARYLOXYALKYL AMMONIUM COMPOUND INVESTIGATED

Number	*Chemical name*	*Formula*
I	1-(2-Phenoxyethyl)-1-ethylpiperidinium bromide	$C_{15}H_{24}BrNO$
II	1-(3-Phenoxypropyl)-1-ethylpiperidinium bromide	$C_{16}H_{26}BrNO$
III	1-(4-Phenoxybutyl)-1-ethylpiperidinium bromide	$C_{17}H_{28}BrNO$
IV	1-(5-Phenoxypentyl)-1-ethylpiperidinium bromide	$C_{18}H_{30}BrNO$
V	1-[4-(2-Methoxyphenoxy)butyl]-1-ethylpiperidinium bromide	$C_{18}H_{30}BrNO_2$
VI	1-[4-(3-Methoxyphenoxy)butyl]-1-ethylpiperidinium bromide	$C_{18}H_{30}BrNO_2$
VII	1-[4-(4-*tert.*-Butylphenoxy)butyl]-1-ethylpiperidinium bromide	$C_{21}H_{36}BrNO$
VIII	1-{4-[4-(1,1,3,3-Tetramethylbutyl)phenoxy]butyl}-1-ethylpiperidinium bromide	$C_{25}H_{44}BrNO$
IX*	1-[4-(4-Nonylphenoxy)butyl]-1-ethylpiperidinium bromide	$C_{26}H_{46}BrNO$
X	1-[4-(2-Biphenyloxy)butyl]-1-ethylpiperidinium bromide	$C_{23}H_{32}BrNO$
XI	1-[4-(4-Biphenyloxy)butyl)-1-ethylpiperidinium bromide	$C_{23}H_{32}BrNO$
XII	1-[4-(1-Naphthyloxy)butyl]-1-ethylpiperidinium bromide	$C_{21}H_{30}BrNO$
XIII	1-[3-(2-Naphthyloxy)propyl]-1-ethylpiperidinium bromide	$C_{20}H_{28}BrNO$
XIV	1-[4-(2-Naphthyloxy)butyl]-1-ethylpiperidinium bromide	$C_{21}H_{30}BrNO$
XV	1-[5-(2-Naphthyloxy)pentyl]-1-ethylpiperidinium bromide	$C_{22}H_{32}BrNO$
XVI	1-[6-(2-Naphthyloxy)hexyl]-1-ethylpiperidinium bromide	$C_{23}H_{34}BrNO$
XVII	1-[8-(2-Naphthyloxy)octyl]-1-ethylpiperidinium bromide	$C_{25}H_{38}BrNO$
XVIII	1-[4-(2-Naphthyloxy)butyl]-1-propylpiperidinium bromide	$C_{22}H_{32}BrNO$
XIX	1-[4-(2-Naphthyloxy)butyl]-1-butylpiperidinium bromide	$C_{23}H_{34}BrNO$
XX	1-[4-(2-Naphthyloxy)butyl]-1-pentylpiperidinium bromide	$C_{24}H_{36}BrNO$
XXI	1-[4-(2-Naphthyloxy)butyl]-1-hexylpiperidinium bromide	$C_{25}H_{38}BrNO$
XXII	1-[4-(2-Naphthyloxy)butyl]-1-cyclohexylpiperidinium bromide	$C_{25}H_{36}BrNO$
XXIII	1-[4-(2-Naphthyloxy)butyl]-1-heptylpiperidinium bromide	$C_{26}H_{40}BrNO$
XXIV	1-[4-(2-Naphthyloxy)butyl]-1-octylpiperidinium bromide	$C_{27}H_{42}BrNO$
XXV	1-[4-(2-Naphthyloxy)butyl]-1-nonylpiperidinium bromide	$C_{28}H_{44}BrNO$
XXVI	1-[4-(2-Naphthyloxy)butyl]-1-decylpiperidinium bromide	$C_{29}H_{46}BrNO$
XXVII	1-[4-(2-Naphthyloxy)butyl]-1-undecylpiperidinium bromide	$C_{30}H_{48}BrNO$
XXVIII	1-[4-(2-Naphthyloxy)butyl]-1-dodecylpiperidinium bromide	$C_{31}H_{50}BrNO$
XXIX	1-[4-(2-Naphthyloxy)butyl]-1-tetradecylpiperidinium bromide	$C_{33}H_{54}BrNO$
XXX	1-[4-(4-*tert.*-Butylphenoxy)butyl]pyridinium bromide	$C_{19}H_{26}BrNO$
XXXI	1-{4-[4-(1,1,3,3-Tetramethylbutyl)phenoxy]butyl}pyridinium bromide	$C_{23}H_{34}BrNO$
XXXII*	1-[4-(4-Nonylphenoxy)butyl]pyridinium bromide	$C_{24}H_{36}BrNO$
XXXIII	1-[4-(2-Naphthyloxy)butyl]pyridinium bromide	$C_{19}H_{20}BrNO$
XXXIV	1-[4-(2-Naphthyloxy)butyl]-2-methylpyridinium bromide	$C_{20}H_{22}BrNO$
XXXV	[4-(4-*tert.*-Butylphenoxy)butyl]benzyldimethylammonium bromide	$C_{23}H_{34}BrNO$
XXXVI	{4-[4-(1,1,3,3-Tetramethylbutyl)phenoxy]butyl}benzyldimethylammonium bromide	$C_{27}H_{42}BrNO$
XXXVII*	[4-(4-Nonylphenoxy)butyl]benzyldimethylammonium bromide	$C_{28}H_{44}BrNO$
XXXVIII	[4-(4-Biphenyloxy)butyl]benzyldimethylammonium bromide	$C_{25}H_{30}BrNO$
XXXIX	[4-(2-Naphthyloxy)butyl]benzyldimethylammonium bromide	$C_{23}H_{28}BrNO$
XL	[4-(4-*tert.*-Butylphenoxy)butyl]trimethylammonium bromide	$C_{17}H_{30}BrNO$
XLI	[4-(2-Naphthyloxy)butyl]trimethylammonium bromide	$C_{17}H_{24}BrNO$
XLII	1-(Propyl)-1-ethylpiperidinium bromide	$C_{10}H_{22}BrN$
XLIII	1-(Butyl)-1-ethylpiperidinium bromide	$C_{11}H_{24}BrN$
XLIV	1-(Pentyl)-1-ethylpiperidinium bromide	$C_{12}H_{26}BrN$
XLV	1-(Hexyl)-1-ethylpiperidinium bromide	$C_{13}H_{28}BrN$
XLVI	1-(Heptyl)-1-ethylpiperidinium bromide	$C_{14}H_{30}BrN$
XLVII	1-(Octyl)-1-ethylpiperidinium bromide	$C_{15}H_{32}BrN$
XLVIII	1-(Nonyl)-1-ethylpiperidinium bromide	$C_{16}H_{34}BrN$
XLIX	1-(Decyl)-1-ethylpiperidinium bromide	$C_{17}H_{34}BrN$

* *p*-Nonylphenol used here could be the mixture of following:



constituting the chromatographic system, the universal Britton–Robinson buffer and other auxiliary chemicals were commercial products of analytical grade purity. Similarly the phenols used for the determination of the group constants of the aromatic part of the molecule of the substances studied, were also commercially available products.

Paper chromatography

A descending chromatographic technique was used with a formamide–1,1′,2,2′-tetrachlorethane system. Both phases of the system were mutually saturated. Prior to impregnation of the paper (Schleicher–Schüll 2043b Mgl) the formamide phase was mixed with methanol in the ratio 1:1. The compounds studied were 25 μg samples of the compounds in ethanolic solution, spotted onto the impregnated chromatogram. The chromatograms were kept in chambers saturated with 1,1′,2,2′-tetrachlorethane. The development period took 4–5 h for the front to run a distance of 40 cm from the start. Compounds with a R_F value lower than 0.1 were chromatographed by using an overrun technique. In this case, compound XX was used for front indication. Chromatograms were detected with Dragendorff's reagent after drying the chromatogram at a temperature which did not exceed 110°. All R_F values listed in Table III are averages from 20 chromatograms, where the deviation of the R_F value from that of control substance XX was not more than $\pm$ 0.03.

Electrophoresis

Electrophoretic mobility was determined on Whatman 31 ET paper. We worked with contact platinum electrodes directly on the electropherogram and no buffer reservoir. Britton and Robinson's universal buffer (0.04 M H_3PO_4, 0.04 M H_3BO_3, 0.04 M CH_3COOH and variable amounts of 0.2 M NaOH) was used adjusted to a constant ionic strength of $\mu = 0.2$ by NaCl. Electrophoresis was performed for 1 h with a potential gradient of 10 V/cm and a temperature of 15°. Tetraethylammonium bromide was used as a standard of mobility. Mobilities u ($cm^2 \cdot V^{-1} \cdot sec^{-1}$) were not corrected for electroosmosis.

RESULTS AND DISCUSSION

From the theoretical concepts of paper chromatography the ΔR_M values for the individual parts of molecules of a substance A can be added according to the formula:

$$R_{M(A)} = x\Delta R_{M(m)} + y\Delta R_{M(n)} + z\Delta R_{M(o)} + \ldots K$$

where:

x, y, z = number of functional groups m, n, o in the substance A,

K = constant for the chromatographic system and paper.

Thus, a prerequisite is given for the study of the relationship between structure and chromatographic behaviour of substances.

In the case of substances of the $(Ar\text{-}O(CH_2)_n\text{–}\overset{|}{\underset{|}{N}}\text{–})^+Br^-$ type we have determined constants for the individual parts of the molecule as indicated in the schematic diagram. The suitability of the chromatographic system used is illustrated by the linear dependence of R_M on the number of carbons in homologous series (Fig. 1).

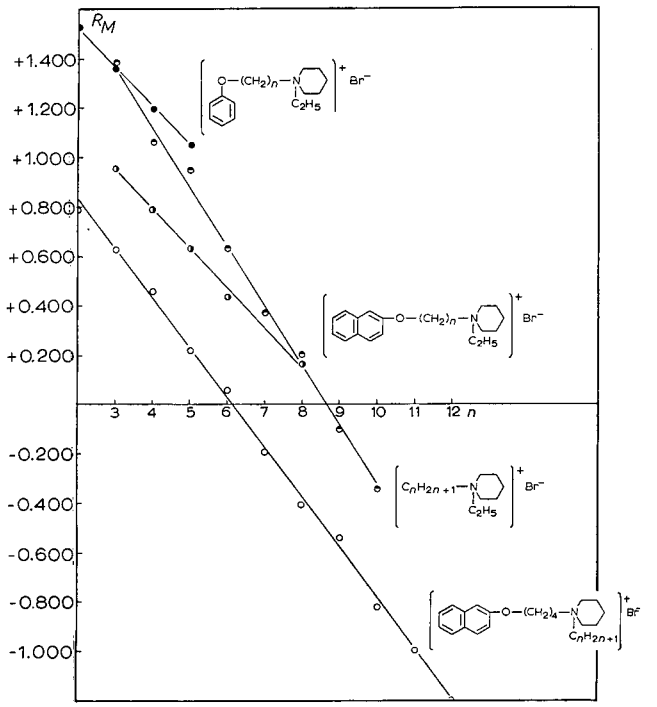


Fig. 1. Dependence of R_M values on the number carbons in an homologous series.

For the aromatic parts of the molecule the group constants (ΔR_M) were computed from the R_F value of relevant phenols according to the formula:

$$\Delta R_M = \log\left(\frac{1}{R_F} - 1\right) - K$$

In this way it is possible to determine the ΔR_M values for all monohydric phenols which display, in the system used, reliable and measurable R_F values. The selection of the phenols (Table II) was determined by the anticipated use of this type of aryloxyalkyl ammonium salt as a solubilizing agent. Further resolution of the aryloxy group into the effect of the substituent, its position etc., would only be of theoretical significance.

Increasing of the number of C atoms in the alkyl substituent on the aromatic nucleus, on the connecting bridge and the nitrogenous part of the molecule influences separation in favour of the non-polar mobile phase (Table III). In an homologous series, C_nH_{2n+1}, where substitution is on the N atom the latter effect is more pronounced than in the case where the series C_nH_{2n} is attached to the connecting bridge. This difference is probably caused by the orientation of the carbon chain of the connecting bridge in the direction of the aromatic nucleus. Such an arrangement in space had been confirmed experimentally in the case of 2-phenoxyethylamine[13] and can be explained as a manifestation of the interaction owing to the varying potential of π–σ bonds[14].

TABLE II

GROUP CONSTANTS

Group	ΔR_M
K-constant for paper and solvent system	−0.024
Carbon atom C_nH_{2n+1}	−0.201
C_nH_{2n}	−0.162
Piperidinium bromide	+2.175
Pyridinium bromide	+2.259
2-Methylpyridinium bromide	+1.963
Benzyldimethylammonium bromide	+1.616
Trimethylammonium bromide	+2.289
Phenoxy	+0.252
2-Methoxyphenoxy	+0.046
3-Methoxyphenoxy	−0.051
2,3-Dimethylphenoxy	−0.407
2,4-Dimethylphenoxy	−0.471
2,5-Dimethylphenoxy	−0.504
2,4,6-Trimethylphenoxy	−0.761
4-*tert.*-Butylphenoxy	−0.683
4-(1,1,3,3-Tetramethyl)phenoxy	−1.143
4-Nonylphenoxy*	−1.380
2-Methyl-5-isopropylphenoxy	−0.850
2-Biphenyloxy	−0.812
4-Biphenyloxy	−0.558
1-Naphthyloxy	−0.371
2-Naphthyloxy	−0.295

* See note to Table I.

Direct determination of group constants for the basic parts of the molecules of the substances studied is impossible in the system used. Data listed in Table II were computed from the differences between the R_M values found and known group constants. Table III shows good agreement of the values found with the ones computed for all compounds studied (with an R_F value not exceeding $\pm$ 0.04). The courses of the mobility curves of the compounds studied (Figs. 2–5) are affected not only by the electromigration phenomena, but also by adsorption on paper. In the case of tetraethylammonium bromide (TEAmmBr), adsorption was not observed.

Measurement of electrophoretic mobility in the series of 2-naphthyloxybutyl nitrogenous quaternary salts (Fig. 2) shows a decrease in mobility in the order trimethylammonium > pyridinium > 2-methylpyridinium > 1-ethylpiperidinium > benzyldimethylammonium. The ΔR_M values for these basic groups also decrease in the same order. Electrophoretic mobility in the series of 1-[4-(2-naphthyloxy)butyl]-1-alkyl piperidinium bromides follows a different course to the mobility of TEAmmBr (Fig. 3). The change in the shape of the curves occurring from C_8 up in the 1-alkylpiperidinium part is caused by the surface activity of these substances (approximatively 40 dyne·cm^{-1} in a concentration of 0.02 *M* (ref. 12)), which is also responsible for the change in the shape of the spots from circular to longitudinal. Lengthening of the connecting carbon bridge between the aromatic part and the basic group results in a decrease of electrophoretic mobility (Fig. 4). Mobilities of 1-alkyl

TABLE III

R_F VALUES OF AMMONIUM COMPOUNDS

Number	*Found*		*Calculated*	
	R_F	R_M	R_F	R_M
I*	0.029	+1.525	0.022	+1.677
II*	0.042	+1.359	0.030	+1.515
III*	0.060	+1.196	0.042	+1.353
IV*	0.082	+1.048	0.059	+1.191
V*	0.10	+0.954	0.08	+1.055
VI*	0.09	+1.005	0.08	+1.050
VII	0.26	+0.454	0.28	+0.418
VIII	0.54	−0.070	0.53	−0.042
IX	0.68	−0.327	0.66	−0.279
X	0.33	+0.308	0.34	+0.289
XI	0.24	+0.501	0.22	+0.543
XII	0.15	+0.753	0.16	+0.730
XIII	0.10	+0.954	0.10	+0.968
XIV	0.14	+0.788	0.14	+0.806
XV	0.19	+0.630	0.19	+0.644
XVI	0.27	+0.432	0.25	+0.482
XVII	0.43	+0.122	0.41	+0.158
XVIII	0.19	+0.630	0.20	+0.605
XIX	0.26	+0.454	0.28	+0.404
XX	0.38	+0.213	0.38	+0.203
XXI	0.47	+0.052	0.50	+0.002
XXII	0.40	+0.176	—	—
XXIII	0.61	−0.194	0.61	−0.199
XXIV	0.72	−0.410	0.72	−0.400
XXV	0.78	−0.545	0.80	−0.601
XXVI	0.87	−0.826	0.87	−0.802
XXVII	0.91	−1.005	0.91	−1.003
XXVIII	0.94	−1.195	0.94	−1.204
XXIX	front	$-\infty$	0.98	−1.606
XXX	0.12	+0.865	0.11	+0.904
XXXI	0.26	+0.454	0.26	+0.444
XXXII	0.34	+0.288	0.38	+0.207
XXXIII	0.06	+1.195	0.05	+1.292
XXXIV	0.09	+1.005	0.08	+1.060
XXXV	0.33	+0.308	0.35	+0.261
XXXVI	0.64	−0.250	0.62	−0.199
XXXVII	0.73	−0.432	0.73	−0.436
XXXVIII	0.25	+0.475	0.24	+0.496
XXXIX	0.20	+0.602	0.20	+0.595
XL*	0.08	+1.061	0.10	+0.934
XLI*	0.06	+1.195	0.05	+1.322
XLII*	0.04	+1.380	0.07	+1.146
XLIII*	0.08	+1.061	0.10	+0.945
XLIV	0.11	+0.954	0.15	+0.744
XLV	0.19	+0.630	0.22	+0.543
XLVI	0.30	+0.368	0.31	+0.342
XLVII	0.39	+0.194	0.42	+0.142
XLVIII	0.56	−0.105	0.53	−0.060
XLIX	0.69	−0.347	0.65	−0.261

* A run-off technique was used.

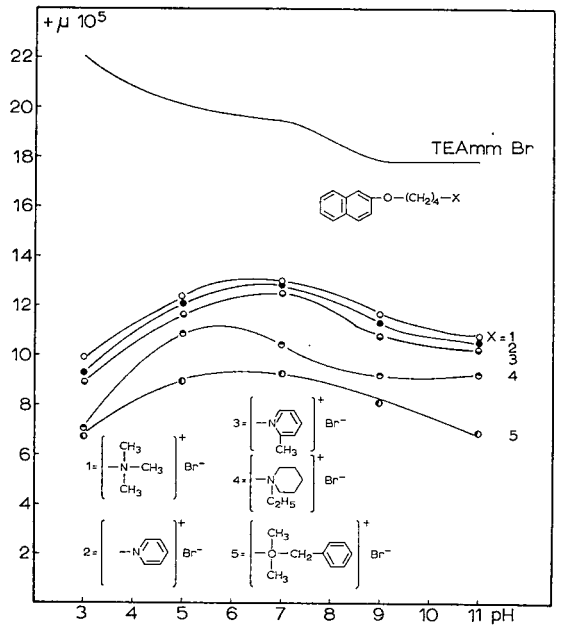


Fig. 2. Electrophoretic mobility of nitrogenous quaternary bromides of general formula as shown.

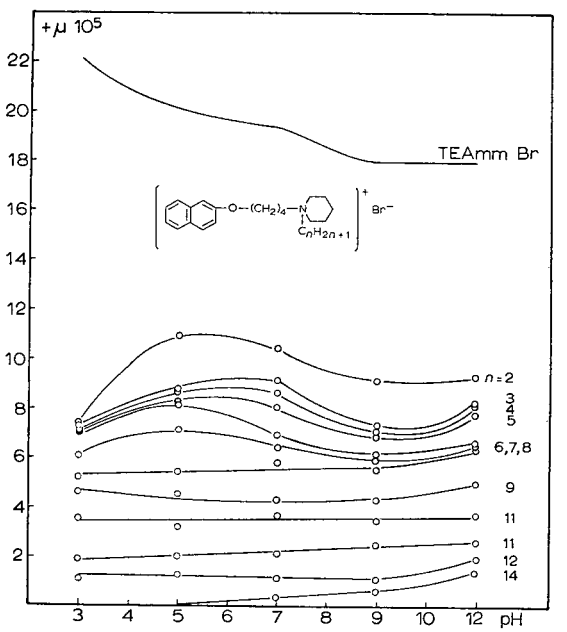


Fig. 3. Electrophoretic mobility of 1-[4-(2-naphthyloxy)butyl]-1-alkyl piperidinium bromides.

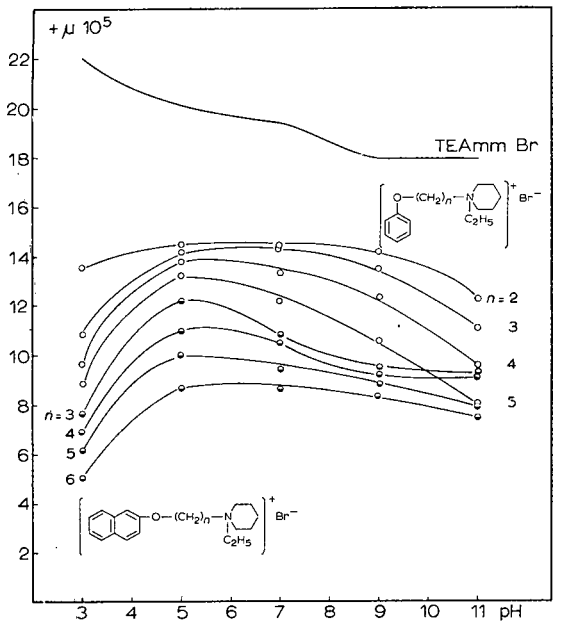


Fig. 4. Effect of chain length on electrophoretic mobility of compounds of general formula as shown.

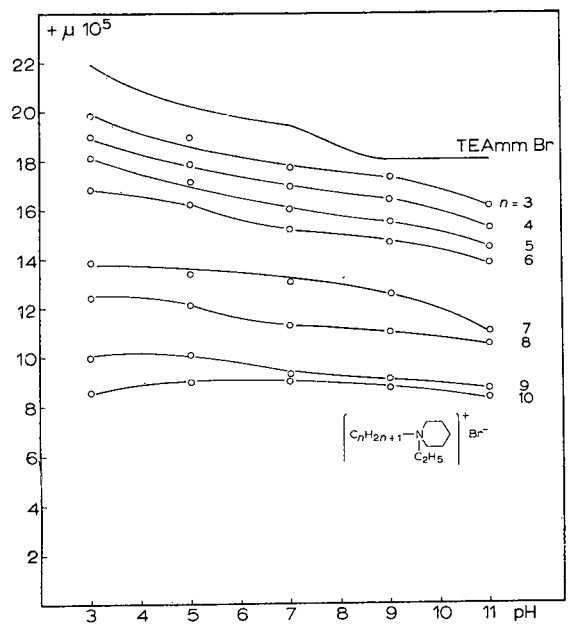


Fig. 5. Electrophoretic mobility of 1-(alkyl)-1-ethylpiperidinium bromides.

ethylpiperidinium bromides with no aryloxy moiety in their molecule (Fig. 5) follow a similar course to TEAmmBr, which is used in the electrophoretic measurements as a standard of mobility.

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Notes

CHROM. 3955

Separation of cyclic-3′,5′-adenosine monophosphate from other adenine nucleotides

Cyclic-3′,5′-adenosine monophosphate (CAMP), an intermediate and a "second messenger" in most of the hormonal reactions[1], has become the subject of increasing interest. The available paper chromatographic methods[2–4] for its separation from other adenine nucleotides are relatively inconvenient and time consuming*. A solvent system consisting of a mixture of isobutyric acid–ammonia–water (64:3:33, v/v) was found by us to be most suitable for the thin-layer chromatographic separation of adenine nucleotides on silica gel.

Materials

The disodium salt of ATP, monosodium salt of ADP, 5′-AMP and adenosine used were products of Nutritional Biochemical Corporation. Cyclic-3′,5′-AMP was a gift sample from Sigma Laboratories. The isobutyric acid was from Riedel (Germany). Isopropanol, ammonia liquor (chromatographic grade) and acetic acid were obtained from British Drug Houses. Silica Gel G (mesh size, 40–80 μ; E. Merck) was employed for thin-layer chromatography (TLC). Dowex 50 X 8 H^+ cation exchanger (mesh size, 200–400; Baker) was used for the column chromatography. The nucleotide spots were visualized under an ultraviolet monochromatolite lamp (Hanovia, Slough, Great Britain). Hilger–Watt (Model FA 41-1/64463) and Cary (−15) recording spectrophotometers were used to read the concentrations of the nucleotides in eluates either from the column or from the thin layers. Double glass-distilled water was used throughout the experiment.

Methods

Thin-layer chromatography. Aqueous solutions of 100–150 μg each of ATP, ADP, 5′-AMP and adenosine and 10–15 μg of CAMP in 10–15 μl were applied to activated, Silica Gel G coated glass plates for TLC. A mixture containing all the nucleotides (20–30 μg of ATP, ADP, 5′-AMP and adenosine and 2–3 μg of CAMP) was also applied. The plates were developed for about 3.5–4 h in the solvent system isobutyric acid–ammonia–water (64:3:33, v/v) at room temperature. The dried plates were exposed to an ultraviolet light source to localise the separated nucleotides. Each spot was then scraped off and extracted with 3 ml of distilled water. The extract was centrifuged at 2500 × g for 20 min, and the supernatant obtained from the extract of each spot was read at its $\lambda_{max.}$ on the spectrophotometers.

The plates were also sprayed with orcinol–H_2SO_4 (ref. 5) reagent and heated

* A separation on DEAE Cellulose layers was reported by YOSHIMOTO *et al.*[12].

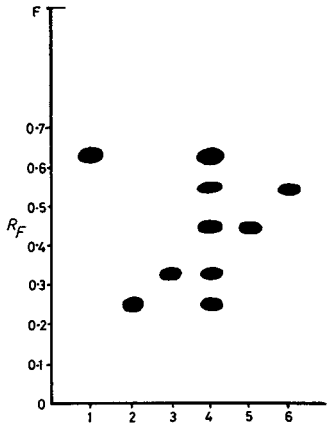

Fig. 1. Thin-layer chromatography on Silica Gel G of adenine nucleotides. 1, Adenosine; 2, ATP; 3, ADP; 4, Mixture of 1, 2, 3, 5, and 6; 5, 5′-AMP; 6, Cyclic-3′,5′-AMP. Solvent system: isobutyric acid–ammonia–water (64:3:33, v/v; pH 4.1). Detection: Monochromatic U.V. light of short wavelength (254 mμ).

in an oven at 110° for 15 min to detect the distinct violet-coloured spots for pentose.

Ion-exchange chromatography. A mixture of ATP, ADP, 5′-AMP, adenosine and CAMP (1250 μg each/ml) was prepared in an aqueous solution containing Tris–HCl buffer (pH 7.3; 8×10^{-2} *M*), $MgSO_4$ (7×10^{-3} *M*) and NaF (2×10^{-2} *M*). Of this mixture 0.2 ml was loaded on the column containing a 50% aqueous suspension of Dowex 50 X 8 H^+ (maximum effective pH, 14.0) plugged with glass wool (resin bed, 0.4–0.6 cm diameter at 8–10 cm resin height, in a total column length of 18–20 cm, with a flow rate of 1 ml/5 min). This was eluted with a total of 9.8 ml distilled water. Three 3-ml eluate fractions were collected separately. On a thin-layer plate, for detection of the nucleotides, 75–100 μl of each was spotted.

Although the $BaSO_4$ adsorption step was not necessary (as seen under *Results*), the eluates were subjected to $BaSO_4$ adsorption according to the method of KRISHNA *et al.*[6]. After $BaSO_4$ treatment the supernatants were spotted on thin-layer plates to check for contamination by other adenine nucleotides. The concentration of CAMP

TABLE I

R_F VALUE OF ADENINE NUCLEOTIDES SEPARATED BY TLC USING SILICA GEL G

Solvent system: isobutyric acid–ammonia–water (64:3:33, v/v; pH 4.1). Detection: Monochromatic U.V. light of short wavelength.

Compound	λ_{max} (mμ) *pH 5.6*	R_F *values*	*Recovery (%)*
ATP	260	0.25 ± 0.01	
ADP	260	0.33 ± 0.01	
5′-AMP	260	0.45 ± 0.01	90 ± 10
Cyclic-3′,5′-AMP	256	0.55 ± 0.01	
Adenosine	259	0.63 ± 0.01	

TABLE II

RECOVERY OF CAMP AFTER ION-EXCHANGE CHROMATOGRAPHY ON A DOWEX 50 X 8 H^+ COLUMN (BEFORE AND AFTER $BaSO_4$ TREATMENT) AS DETECTED BY TLC

Nucleotides	*Effluents from the Dowex column*			*Recovery of CAMP from column (%)*
	I	*II*	*III*	
ATP	—	—	—	—
ADP	—	—	—	—
5′-AMP	—	—	—	—
Cyclic-3′,5′-AMP	—	—	+	80 ± 20
Adenosine	—	—	—	—

in the fractions eluted from the column and from the thin-layer plates was determined by the spectrophotometer at its $\lambda_{max.}$

Results

With the solvent used for TLC, CAMP is distinctly separated from other adenine nucleotides (Fig. 1). Table I represents the R_F values obtained for individual nucleotides in such a system. Percentage recovery of the nucleotides from the thin-layer plates as determined spectrophotometrically was 90 ± 10.

In order to check the recovery of the nucleotides, a known amount of the mixture of adenine nucleotides (1250 μg/ml each) was processed according to the method of KRISHNA *et al.*[6], involving separation of the mixture on a Dowex-50 X 8 H^+ column followed by $BaSO_4$ adsorption. The eluates from the column (as described in *Methods*) before and after treatment with $BaSO_4$ were scanned after TLC for nucleotide content (Table II). It is evident from Table II that only CAMP is eluted from the column, while other adenine nucleotides were not detected when eluates were checked on thin-layer plates. Almost 80 ± 20% of the CAMP could be recovered. Since other nucleotides were found to be absent from the column eluates, the $BaSO_4$ adsorption step used in KRISHNA's method could conveniently be omitted. However, to confirm our findings, the three fractions obtained from the column were treated with $BaSO_4$, and the supernatant from each was analysed for CAMP. Again, only the third fraction was CAMP positive. This spot on thin-layer plates, when eluted with water and read at 256 mμ on the spectrophotometer, gave the same recovery (90 ± 10%).

Discussion

In a series of publications from different laboratories[7,8] radioactive material was used for the assay of adenyl cyclase in terms of CAMP formed from the reaction mixtures. CAMP was also quantitated by the degree of conversion of inactive phosphorylase *b* to active phosphorylase *a*[9,10].

Paper and column chromatographic methods[2–4,6] have also been used for the isolation and quantitation of CAMP in the reaction mixtures.

The available paper chromatographic methods have employed the following solvent systems for the separation of CAMP:

isopropanol–ammonia–water (70:20:10, v/v)
isopropanol–acetic acid–water (60:30:10, v/v)
isobutyric acid–ammonia–water (66:1:33, v/v)
isobutyric acid–0.5 *M* NH_4OH (10:6, v/v).

All these solvent systems were tried on paper as well as on thin layers, but were found to be time consuming and unsatisfactory.

According to our procedure, CAMP can be eluted from the Dowex 50 X 8 H^+ column without the interference of other nucleotides in the reaction mixture. The $BaSO_4$ adsorption step is therefore unnecessary. This is probably due to the weak chelating property of CAMP as compared to other adenine nucleotides[11].

Moreover, the thin-layer chromatographic system separates all the nucleotides very distinctly, and hence CAMP could be assayed conveniently from the biological system within a short period.

Further work on quantitation of CAMP using Bial's reagent (orcinol–HCl) is in progress.

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CHROM. 3942

Estimation of molecular weights of proteins by agarose gel filtration

At the present time gel filtration is widely used, as both an analytical and a preparative tool, for the fractionation, isolation and purification of proteins, enzymes, hormones, antibiotics, nucleic acids, etc. DETERMANN's[1] monograph has exhaustively reviewed the literature concerning this subject.

Gel filtration on agarose has recently been introduced[2,3]. The use of agarose gel beads for chromatographic molecular sieving of proteins of high molecular weights has proven to have advantages over the use of other gels. Furthermore, these gels make it possible to extend the working range up to limits which are far beyond those of dextran and polyacrylamide gels.

Among the principal features of filtration with agarose bead gels are the high flow rates which are obtained and the absence of the packing procedure.

The present paper describes a procedure for the evaluation of molecular weights of proteins and the splitting off of a "nucleoprotein-enzyme complex" by means of an agarose gel. Comparative studies with two commercial types of agarose gels are also reported.

Materials and methods

Proteins used in this study are listed in Table I. Aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase, EC 4.2.1.24) from soybean callus tissue system was

TABLE I

PROTEINS EMPLOYED IN THE STUDY

Protein	*Source*	*Mol. wt.* $\times 10^{-3}$	*Reference*	*Amounts used (mg)*
Thyroglobulin (bovine) type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	670	EDELHOCH[4]	8–15
Fibrinogen (bovine) fraction I citrated type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	330–340	SHULMAN[5]	10–30
Catalase (from ox liver) purified powder	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	230–250	SAMEJIMA AND YANG[6]	10–20
γ-Globulins (bovine) Cohn Fraction II	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	150–170	PHELPS AND PUTNAM[7]	10–20
Serum albumin dimer (present in bovine serum albumin)		134		(2 × mol. weight of serum albumin)
Serum albumin bovine, fraction V from bovine plasma	Armour Pharmaceutical Co. Ltd., Chicago, Ill., U.S.A.	67	PHELPS AND PUTNAM[7]	10–20
Ovoalbumin	Schering-Kahlbaum AG, Berlin	45	WARNER[8]	10–20
Myoglobin (sperm whale)	L. Light & Co. Ltd., Colnbrook, Bucks., England	17.8	EDMUNDSON AND HIRS[9]	5–10
Cytochrome c from horse heart, type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	12.4	MARGOLIASH[10]	4–7

partially purified, and its activity was determined according to TIGIER *et al.*[11]. Blue Dextran 2000 (containing some material of very high molecular weight) and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio Gel-A 1.5 m was the generous gift of Bio Rad Laboratories. Richmond, Calif., U.S.A.

SR 25/45 columns from Pharmacia Fine Chemicals, Uppsala, Sweden, and vertical glass tubes (60 cm long; 2.5 cm I.D.) equipped with capillary outlets were used. In the latter a disc of polypropylene tissue was used as bed support.

Sepharose 4B and Bio Gel-A 1.5 m, the agarose gels used in this work, were obtained as a slurry in distilled water or Tris buffer containing 0.02% sodium azide as a bacteriostatic agent. Each column was filled with buffer, and the gel, previously deaerated under reduced pressure, was poured into the column by using an extension tube. The head of water was gradually increased until reaching a pressure of 30 to 45 cm of level difference. Operating flow rates were between 25 to 35 ml/h. The columns were ready for use after equilibrating with two bed volumes of the appropriate buffer. This was done either with 0.05 *M* Tris–HCl buffer (pH 7.4) or 0.1 *M* glycine–NaOH buffer (pH 9.0) unless otherwise stated.

All experiments were done at room temperature or at 4°; no significative differences in elution volumes were observed at either temperature. Samples were dissolved in the elution buffer and applied to the top of the column by layering under the solution already present. Sucrose was added, when necessary, in order to increase solution density.

Column eluates were collected with an automatic fraction collector (LKB, Stockholm, Sweden) equipped with a Uvicord I effluent monitor and chopper bar recorder, registering light absorption at 254 nm. When columns were run at 4°, a Colora low-temperature bath (KT 305, Lorch, Württenberg, Germany) was used.

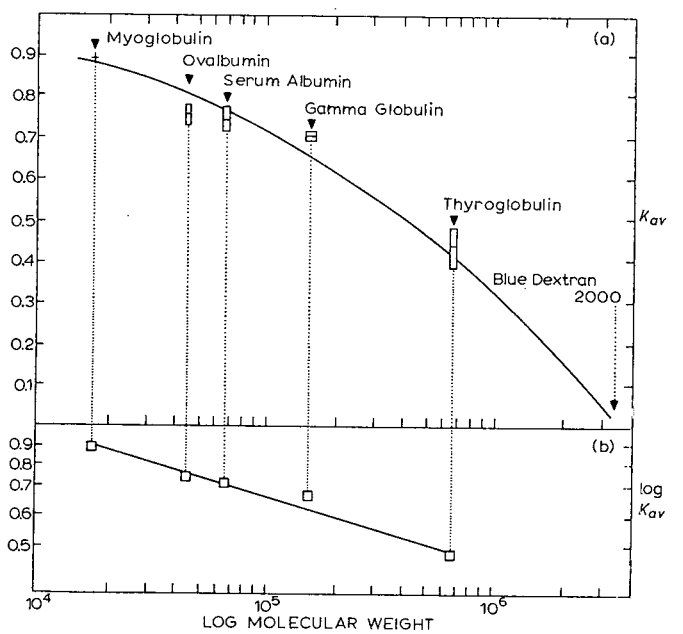

Fig. 1. (a) Plots of K_{av} against log(mol. wt.) and (b) plots of log K_{av} against log (mol. wt.) for proteins on Sepharose 4B.

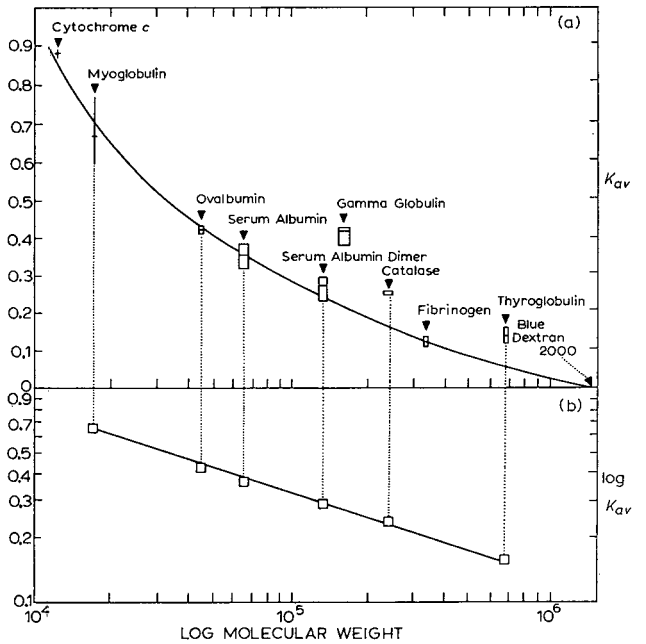

Fig. 2. (a) Plots of K_{av} against log(mol. wt.) and (b) plots of log K_{av} against log(mol. wt.) for proteins on Bio Gel-A 1.5 m.

Results and discussion

The elution behaviour of a substance in gel chromatography can be described by different variables which are derived from the elution volume. These relationships have been compiled by DETERMANN[12]; some of them are empirical and some were derived on the basis of theoretical considerations.

A magnitude independent of the geometry and packing density of the column and easily measured, defined by LAURENT AND KILLANDER[13] as K_{av}, was plotted versus the log of the molecular weight. Fig. 1a and 2a show plots of K_{av} values from Bio Gel-A 1.5 m and Sepharose 4B columns versus the log of the molecular weight for a set of standards.

Differences in the preparation of these agarose gels and/or raw materials might account for the different shapes of these curves. Furthermore, even though the non-linearity of these relationships is still an open question, a possible reason for this might be found in the intrinsic nature of agarose gels, which are known to possess no cross-linking, as dextran and polyacrylamide gels do, but rather hydrogen bonding between different portions of a chain or between different chains.

From Figs. 1a and 2a, it is clear that Sepharose 4B provided better resolution for proteins in the high molecular weight zone, whereas Bio Gel-A 1.5 m would be better for the lower zone, even though the latter provided a wide working range.

As has been mentioned, a non-linear dependence of the K_{av} (or elution volume) or the log of the molecular weight was observed for agarose gels; but we have found that a straight line is obtained if the log of K_{av} is plotted against the log of the molecular weight, as shown in Figs. 1b and 2b.

As it has already been pointed out for other gels, some proteins also exhibited abnormal behaviour on these agarose gels.

Carbohydrate content, differences in shape of native proteins and uncertainty regarding the state of aggregation[1,14–17] might account for this fact. In addition, some proteins deviated to a different extent on each agarose gel; for example, fibrinogen exhibited a normal behaviour on Bio Gel-A 1.5 m, whereas variable values of K_{av} were obtained on Sepharose 4B. On the other hand, γ-globulin and thyroglobulin are retarded on Bio Gel-A 1.5 m, particularly the former, which deviated to a lesser extent on Sepharose 4B; thyroglobulin, however, fitted the curve. Moreover, the observed values for catalase, especially on Sepharose 4B at different pH values (not shown in Fig. 1a) would suggest some variation in its state of aggregation[18].

Further purification of aminolaevulinate dehydratase

When aminolaevulinate dehydratase, obtained as mentioned above, was run on Sephadex G-200 or Bio Gel P-300 columns, it was systematically eluted with the void volume. Evidence was obtained which suggested that the enzyme was associated with a nucleoprotein; therefore, attempts were made to separate the components. Among them, protamin sulphate treatment was performed, but the resulting supernatant was absolutely devoid of activity and all efforts to split the "enzyme–nucleoprotein complex" were unsuccessful[19].

When preparations of such a complex were run on Sepharose 4B columns at 4°, with 0.1 *M* glycine–NaOH buffer (pH 9.0) as elution buffer, three peaks were obtained (Fig. 3); the first one was eluted with the void volume and contained the unwanted

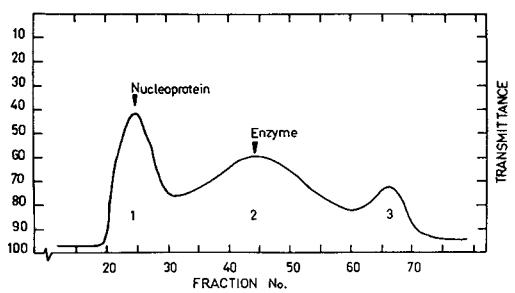

Fig. 3. Gel filtration on Sepharose 4B of a "nucleoprotein–aminolaevulinate complex" from a soybean callus tissue system. 8–10 ml of solution in 0.1 *M* glycine–NaOH buffer (pH 9.0) containing approx. 80 mg of protein were applied to a SR 25/45 Pharmacia column of Sepharose 4B equilibrated with the same buffer. Flow rate: 0.5 ml/min. Peak 1 was eluted with the void volume and contained the unwanted nucleoprotein; all enzymic activity was associated with the second protein peak.

nucleoproteins, while all the enzymic activity was associated with the second peak. Thus, this technique allowed a further purification of the enzyme, yielding a 40-fold over-all enrichment.

By running the highly purified enzyme through calibrated columns of Sepharose 4B, Sephadex G-200 and Bio Gel P-300, an estimated molecular weight of 280,000 was obtained.

Attempts to dissociate the complex into its nucleoprotein and enzyme com-

ponents on Bio Gel-A 1.5 m columns with different buffers containing 2 *M* NaCl[20] were unsuccessful. A single wide peak eluted with the void volume was always obtained.

Summing up, the use of column chromatography on agarose gels offers advantages over dextran and polyacrylamide gels, permitting the estimation of molecular weights of proteins within wider molecular weight ranges, the use of high flow rates and the absence of packing of the column. Moreover, gel filtration on Sepharose allows a good and rapid separation of nucleoproteins (or nucleic acids) and proteins from a complex of both, while with dextran or acrylamide gels it is difficult to dissociate those components.

Finally, the present methods of application have the advantages of simplicity, speed and fairly good reproducibility.

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CHROM. 3952

The identification of the free carbohydrates present in some grass pollens*

Since grass pollen allergens have been reported variously as carbohydrate[1,2] and protein[3–7], it was considered pertinent to investigate the free sugar content of aqueous extracts of some grass pollens. The free amino acid content of these pollens has been reported previously[8]. Furthermore, although pollen may be in the atmosphere for a considerable time, hay fever sufferers normally associate the severity of their attacks with the time when pollen is shed from the anthers and since at this time the pollen grains are potentially viable, extracts of both viable and non-viable pollens[9] were examined to ascertain whether there was any difference with respect to their free sugar content.

Although numerous sugars[10–16] and sugar alcohols[11,17–22] have been reported in various pollens the only reports on sugars present in grass pollens are given by GOUGH[23] and AUGUSTIN[13,22]. The former found a complex carbohydrate yielding on acid hydrolysis, arabinose, galactose and a non-reducing sugar in *Phleum pratense* L. pollen while the latter reported the presence of glucose, mannose or fructose and inositol in *Dactylis glomerata* L. and *Phleum pratense* L. pollens. No experimental evidence was presented by AUGUSTIN[13] for the resolution of the "mannose–fructose' spot although a claim is made in the discussion that extracts of *P. pratense* and *D. glomerata* pollens contain free glucose, fructose and inositol.

Experimental

The grasses examined and details of their collection and extraction procedure have been reported previously[8]. A summary of the experimental procedures for thin-layer chromatography (TLC) is given in Table I.

TABLE I

SUMMARY OF EXPERIMENTAL PROCEDURES FOR TLC

Adsorbent	Silica gel, buffered with boric acid	Cellulose
Thickness	250 μ	250 μ
Activation	Air dried, 1 h; 110° for 60 min	105° for 10 min.
Solvent system	(I) Methyl ethyl ketone–glacial acetic acid–methanol (3:1:1)	(II) Ethyl acetate–pyridine–water (12:5:4)
Method	Ascending in saturated chamber	
Temperature	20–22°	
Distance	10 cm	
Load	Pollen extracts 2 μl (original extracts diluted 2 in 5 to give a glycerol concentration of 20%) Reference sugars 2 μl (1% solutions of reference compounds in 20% glycerol)	
Detection	Sugars: Anisaldehyde–sulphuric acid[24] Naphthoresorcinol–phosphoric acid[25] Inositol: Silver nitrate and sodium hydroxide solution[26]	

* This work forms part of a thesis submitted by G. H. JOLLIFFE for a Ph.D. degree of the University of London, May, 1967.

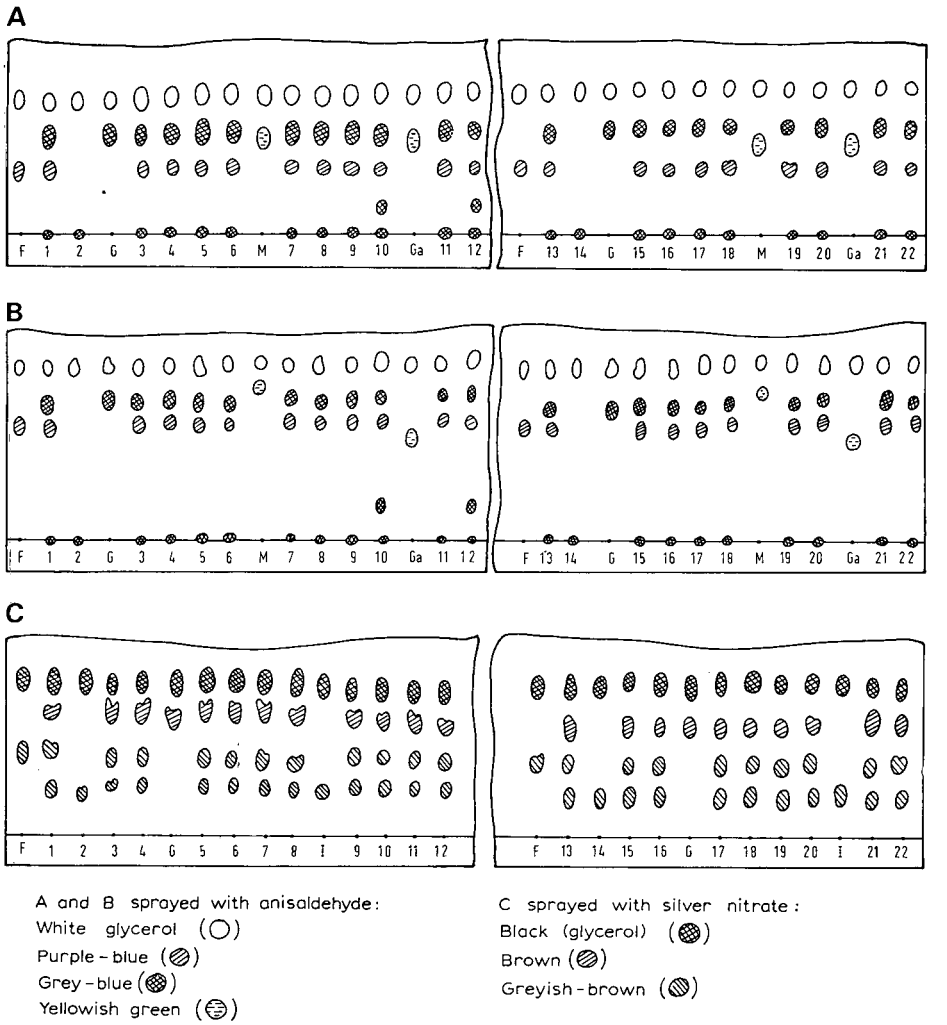

Fig. 1. Tracings of chromatograms. A and C silica gel buffered with boric acid, solvent system I; B cellulose, solvent system II. A and B sprayed with anisaldehyde[24]; C sprayed with silver nitrate[26]. Reference carbohydrates dissolved in 20% glycerol, fructose (F), glucose (G), mannose (M), galactose (Ga), *meso*-inositol (I). Pollen extracts (glycerol concentration 20%): 1, *Anthoxanthum odoratum* L. viable (V), 2, non-viable (N); 3, *Poa trivialis* L. (V), 4, (N); 5, *Dactylis glomerata* L. (V), 6, (N); 7, *Lolium perenne* L. (V), 8, (N); 9, *Alopecurus pratensis* L. (V), 10, (N); 11, *Festuca pratensis* Huds. (V), 12, (N); 13, *Cynosurus cristatus* L. (V); 14, (N); 15, *Arrhenatherum elatius* (L.) J. and C. Presl. (V), 16, (N); 17, *Holcus lanatus* L. (V), 18, (N); 19, *Agrostis tenuis* Sibth (V), 20, (N); 21, *Phleum pratense* L. (V), 22, (N).

Results and discussion

The influence of glycerol on the movement of some simple sugars on silica gel and cellulose thin layers has been reported[27]. Preparation of the reference solutions in 20% glycerol and dilution of the pollen extracts to give a glycerol concentration of 20% were found satisfactory for this work. The results obtained are shown in Table II and Fig. 1.

TABLE II

SUMMARY OF RESULTS OF INVESTIGATION OF POLLEN EXTRACTS FOR FREE SUGARS AND *meso*-INOSITOL

(Layer: Silica gel, buffered with boric acid; Solvent system: Methylethyl ketone–glacial acid–water, 3:1:1)

Pollen extract		*Carbohydrates detected*			
		Fructose	*Glucose*	*Meso-inositol*	*hR_F values of additional components*
Anthoxanthum odoratum L.	V	+	+	+	○
	N	−	−	+	○
Poa trivialis L.	V	+	+	+	○
	N	+	+	+	○
Dactylis glomerata L.	V	+	+	+	○
	N	+	+	+	○
Lolium perenne L.	V	+	+	+	○
	N	+	+	+	○
Alopecurus pratensis L.	V	+	+	+	○
	N	+	+	+	○; 9*
Festuca pratensis Huds.	V	+	+	+	○
	N	+	+	+	○; 9*
Cynosurus cristatus L.	V	+	+	+	○
	N	−	−	+	○
Arrhenatherum elatius (L.) J. and C. Presl	V	+	+	+	○
	N	+	+	+	○
Holcus lanatus L.	V	+	+	+	○
	N	+	+	+	○
Agrostis tenuis Sibth.	V	+	+	+	○
	N	+	+	+	○
Phleum pratense L.	V	+	+	+	○
	N	+	+	+	○

+ = sugar detected; − = sugar not detected.
V = viable pollen extract; N = non-viable pollen extract.
* hR_F of this sugar when chromatographed on a cellulose thin layer using ethyl acetate–pyridine–water (12:5:4) is 11.

Fig. 1 shows that in addition to the uppermost white spot which corresponds with the position of the glycerol reference, the extracts, in general, contain two components and a base-line fraction which yield coloured compounds on spraying.

The rate of movement of the reference galactose, mannose and glucose is similar and while there could be difficulty in distinguishing these sugars by colour after spraying with naphthoresorcinol–phosphoric acid (all give blue colours), galactose and mannose yield a yellow/green colour whereas glucose gives a grey/blue colour when sprayed with the anisaldehyde reagent. There was no evidence of a yellow/green colour in the extracts examined. (Both in rate of movement and in colour after spraying the upper coloured spot corresponds with the reference glucose while the lower coloured spot corresponds with fructose.) Confirmation of the absence of galactose and mannose was obtained from their differential movement on cellulose layers (Fig. 1B). Although AUGUSTIN[13] had difficulty in resolving the "mannose–fructose" spot using paper chromatography, no difficulty was experienced using the thin-layer technique described. Furthermore, AUGUSTIN[13] reported "the lowest spot

appeared in the mannose–fructose position, its colour with naphthoresorcinol (red) placed it in the keto–hexose group". It would, therefore, appear from the facts she presented that the sugar was not mannose but fructose. The former yields a blue colour with naphthoresorcinol whereas the latter gives a red/purple colour[25,28].

All the viable pollen extracts examined showed the presence of fructose, glucose and a base-line fraction (Table II). Variation was found, however, in the extracts from non-viable pollen. Whereas only the base-line fraction was detected in *Anthoxanthum odoratum* and *Cynosurus cristatus*, the *Alopecurus pratensis* and *Festuca pratensis* extracts revealed one additional sugar not present in the viable extracts. This latter sugar gave a blue colour with naphthoresorcinol–phosphoric acid indicating it to be an aldohexose.

The base-line fraction was found to be a non-reducing carbohydrate (Fig. 1C) since it did not reduce the silver nitrate reagent used for the detection of *meso*-inositol. This together with its immobility suggested it may be a polysaccharide. After hydrolysis, with dilute sulphuric acid, of the base-line fraction only glucose (Table II) was detected indicating that the polysaccharide was built up of glucose units.

AUGUSTIN[22] had already shown, by a microbiological assay technique, that *Dactylis glomerata*, *Alopecurus pratensis* and *Anthoxanthum odoratum* pollens contained free *meso*-inositol. Fig. 1C and the results summarised in Table II confirm her findings and show that this polyhydric alcohol is found in all the grass pollen extracts examined.

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CHROM. 3938

Über die Trennung stereoisomerer 2,4-Dinitrophenylhydrazone parasubstituierter Benzophenone

Unterschiedliche Schmelzpunkt-Angaben bei 2,4-Dinitrophenylhydrazonen von Aldehyden und unsymmetrisch substituierten Ketonen veranlassten THEILACKER[1] zu Untersuchungen über mögliche Ursachen, da angenommen werden konnte, dass die Differenzen nicht immer durch unreine Substanzen hervorgerufen wurden. Es gelang ihm, die zwei stereoisomeren 2,4-Dinitrophenylhydrazone, im folgenden 2,4-DNPH, des *p*-Chlorbenzophenons zu isolieren, die deutliche Schmelzpunktunterschiede zeigten. In diesem Zusammenhang durchgeführte Versuche, das Isomeren-Gemisch durch chromatographische Adsorption an Aluminiumoxid zu trennen, blieben erfolglos. Nachdem EDWARDS[2] durch Mehrfach-Dünnschichtchromatographie mit Phasenumkehr bei 2,4-DNPH aliphatischer Aldehyde die isomeren syn- und anti-Formen trennen konnte und TSCHETTER[3] und REIMANN[4] die isomeren 2,4-DNPH unsymmetrisch substituierter Benzophenone an Kieselgel mit Chloroform bzw. Benzol trennten, gelang uns die Trennung stereoisomerer 2,4-DNPH einiger weiterer monosubstituierter Benzophenone in *para*-Stellung durch Mehrfach-Dünnschichtchromatographie.

Experimentelles

Für die Versuche standen Benzophenone zur Verfügung, die an einem Phenylkern in *para*-Stellung Fluor, Chlor bzw. Brom oder eine Hydroxyl-, Methyl- bzw. Äthyl-Gruppe als Substituenten enthielten. Durch Umsetzen von jeweils 0.5 g der Ketone mit 0.8 g 2,4-Dinitrophenylhydrazin in 50 ml siedendem Äthanol unter Zusatz von 4 ml konzentrierter Schwefelsäure sind die 2,4-DNPH-Derivate hergestellt worden.

Zur Sicherung der Ergebnisse sind als Modellsubstanzen die stereoisomeren 2,4-DNPH des *p*-Chlor-benzophenons nach der von THEILACKER[1] beschriebenen Arbeitsweise aus dem Reaktionsgemisch isoliert und als Vergleichssubstanzen einzeln und im Gemisch chromatographiert worden. Ausserdem sind durch präparative Dünnschichtchromatographie diese Stereoisomeren getrennt, aus der Schicht mit Chloroform eluiert und nach Umkristallisieren aus Chloroform durch Schmelzpunkt-Bestimmung charakterisiert worden.

Die dünnschichtchromatographischen Trennungen der Reaktionsprodukte erfolgten an DC-Fertigplatten Kieselgel F_{254} (Merck), die mit einer Lösung von Dimethylformamid in Aceton imprägniert waren.

Bei einer Laufstrecke von 10 cm liessen sich die Halogen-Substitutionsprodukte mit Cyclohexan als Fliessmittel nach sechsmaliger Mehrfach-Chromatographie an Schichten, imprägniert mit Dimethylformamid–Aceton (1:1), in die beiden isomeren Formen auftrennen. Die Isomeren-Trennung der Hydroxy-Verbindung gelang bereits nach dem 2. Lauf mit dem Fliessmittel-Gemisch Cyclohexan–Benzol (2:1). In diesem Fall waren die Kieselgelplatten mit einem Dimethylformamid–Aceton-Gemisch im Verhältnis 1:3 imprägniert worden. Entsprechend vorbehandelte Platten dienten auch zur Chromatographie der Methyl- und Äthyl-Derivate. Im Gegensatz zu den übrigen Verbindungen konnte keine deutliche Isomeren-Trennung beobachtet werden. Lediglich beim 4-Äthyl-benzophenon-2,4-DNPH war nach dem 4. Lauf mit Cyclohexan als Fliessmittel eine Isomeren-Trennung schwach erkennbar, die aber durch weitere Mehrfach-Chromatographie nicht verbessert werden konnte.

Ergebnisse und Diskussion

Wie sich bei zahlreichen Versuchen zur dünnschichtchromatographischen Trennung *cis–trans*-isomerer Verbindungen herausgestellt hat, wandert jeweils die *trans*-Form vor der *cis*-Form. Ein solches Verhalten zeigen *cis–trans*-isomere Carbonsäuren[5], langkettige aliphatische Fettsäure-Derivate[6], Cyclopentandiole[7], 1-Amino-2-hydroxy-indan[8] und die Azobenzole[9], um nur einige Beispiele zu nennen.

Entsprechend verhalten sich auch die im Rahmen dieser Arbeit chromatographierten Benzophenon-Derivate. Wie bei den von EDWARDS[2] untersuchten aliphatischen Aldehyd-2,4-DNPH wandert auch bei den unsymmetrisch substituierten Keton-2,4-DNPH die *syn*-Form hinter der *anti*-Form auf dem Chromatogramm. Aus der Fig. 1 lässt sich ausserdem die Tendenz erkennen, dass sich mit zunehmendem Atomgewicht der Halogen-Substituenten die Wanderungsstrecken verlängern. Dieses

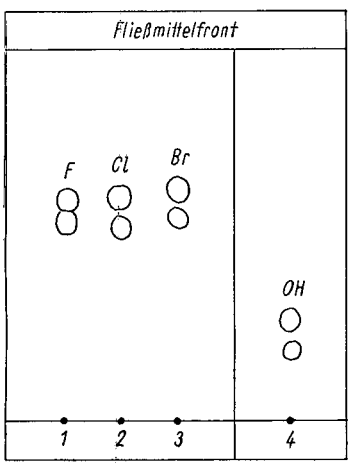

Fig. 1. Trennung der stereoisomeren 2,4-Dinitrophenylhydrazone von *p*-Fluor-, *p*-Chlor- und *p*-Brombenzophenon (1,2,3) nach sechs Läufen (Fliessmittel: Cyclohexan) und von *p*-Hydroxybenzophenon (4) nach zwei Läufen (Fliessmittel: Cyclohexan–Benzol, 2:1).

Ergebnis stimmt mit früheren Beobachtungen[10] ebenso überein wie das Verhalten der Alkyl-Substitutionsprodukte, indem das 4-Äthyl-benzophenon-2,4-DNPH vor dem entsprechenden 4-Methyl-Derivat wandert. Verglichen mit den anderen Verbindungen konnten bei den Alkyl-Substitutions-Produkten keine befriedigenden Isomeren-Trennungen erzielt werden. Es kann aber angenommen werden, dass von einer bestimmten Kettenlänge der Substituenten an auch hier *syn*- und *anti*-Form dünnschichtchromatographisch zu trennen sind.

Auffallend schnell erfolgte die Auftrennung der Isomeren beim 4-Hydroxybenzophenon-2,4-DNPH. Bereits nach zwei Läufen mit dem Fliessmittel-System Cyclohexan–Benzol (2:1) hat sich die *anti*-Form klar von der *syn*-Form abgesetzt (Fig. 1). Der Trenn-Effekt lässt sich sogar noch vergrössern, wenn die Fliessmittel-Zusammensetzung zu gleichen Teilen aus Cyclohexan-Benzol besteht. Allerdings beobachten wir dabei eine schlechtere Reproduzierbarkeit.

Abschliessend sei erwähnt, dass der Anteil an Dimethylformamid in der Schicht von Einfluss auf die Isomeren-Trennungen ist.

Dank

Herrn U. GERMER danke ich für die wertvolle Mitarbeit bei den chromatographischen Trennungen.

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CHROM. 3930

Thin-layer chromatography of aldehyde hydrazones of 2-hydrazinobenzothiazole

Thin-layer chromatographic (TLC) methods for the identification of carbonyls using the separation of 2,4-dinitrophenylhydrazones do not readily distinguish between aldehydes and ketones other than by differences in R_F values. A previous paper[1] described a TLC method for the separation of aliphatic aldehydes in the presence of ketones using azine derivatives of 3-methyl-2-benzothiazolone hydrazone. Aromatic and aliphatic aldehydes can be present together in complex mixtures and a method for identifying them in the presence of ketones is desirable. Such a method is described in this work.

The reactions involved are firstly between an aldehyde and 2-hydrazinobenzothiazole to form a hydrazone. This hydrazone reacts with *p*-nitrobenzenediazonium fluoborate to form a coloured dye which reacts in turn with a base to form a blue or green anion. These reactions were described by SAWICKI AND STANLEY[2] as a test for aliphatic, aromatic and heterocyclic aldehydes. For the purposes of TLC identification the aldehyde hydrazones of 2-hydrazinobenzothiazole are chromatographed on silica gel. Detection is by successive spraying with the *p*-nitrobenzenediazonium fluoborate reagent and alcoholic KOH. The final spraying with base is necessary as a confirming test because the colours formed with the diazonium reagent are not specific for the hydrazones. Ketones form hydrazones with 2-hydrazinobenzothiazole, however the hydrazones do not form coloured compounds with the diazonium reagent or by subsequent treatment with base.

Experimental

Apparatus and reagents. E. Merck precoated Silica Gel F_{254} TLC plates with a layer thickness of 250 μ were used for the investigation. 2-Hydrazinobenzothiazole and *p*-nitrobenzenediazonium fluoborate were obtained from Distillation Products Industries, Rochester 3, N.Y., U.S.A.

Preparation of hydrazones. One mmole of the aldehyde was added to 30 ml of ethanol containing 1 mmole of 2-hydrazinobenzothiazole and a drop of glacial acetic acid. The mixture was warmed under reflux on a steam bath for 30 min to 1 h. The hydrazone was obtained from the reaction mixture by cooling in an ice bath and if necessary by the addition of water. The hydrazones of citral and isovaleraldehyde were obtained by pouring the reaction mixture into ice water. The derivatives were recrystallised from ethanol–water mixtures. Chloroform or ethyl acetate solutions of the hydrazones were stored under refrigeration.

Plate preparation. Silica Gel F_{254} plates were activated at 110° for 1 h and then stored over blue silica gel in a desiccator.

Thin-layer chromatography. Chloroform or ethyl acetate solutions of the hydrazones were applied to a start line 2 cm from the bottom of a TLC plate. After 1 h equilibration of the spotted plate in a solvent vapour saturated chamber, development was performed with a solvent system of light petroleum (b.p. 30–40°)–ethyl acetate–acetic acid (88:10:2). After the front had advanced 15 cm from the start-line, the plate was air dried.

TABLE I

R_F VALUES AND COLOURS OBTAINED WITH SPRAY REAGENTS FOR ALDEHYDE HYDRAZONES OF 2-HYDRAZINOBENZOTHIAZOLE

Aldehyde hydrazone	R_F *value*	*Colour*	
		With p-nitrobenzenediazonium fluoborate	*With p-nitrobenzenediazonium fluoborate, then alcoholic KOH or NH_3 vapour*
Formaldehyde	0.35	orange	blue
Acetaldehyde	0.40	orange	blue
Propionaldehyde	0.44	orange	blue
Butyraldehyde	0.50	orange	blue
Isobutyraldehyde	0.48	purple	blue
2-Methylbutyraldehyde	0.52	purple	blue
Hexyl aldehyde	0.55	orange	blue
Heptyl aldehyde	0.56	orange	blue
Octyl aldehyde	0.57	orange	blue
Nonyl aldehyde	0.58	orange	blue
Benzaldehyde	0.38	purple	blue
p-Tolualdehyde	0.36	purple	blue
Cuminyl aldehyde	0.44	purple	blue
Phenylacetaldehyde	0.40	orange	blue
Hydrotropaldehyde	0.39	orange	blue
Cinnamaldehyde	0.38	orange	green
Hydrocinnamaldehyde	0.36	orange	blue
α-amylcinnamaldehyde	0.52	orange	blue
α-Hexylcinnamaldehyde	0.51	orange	blue
Citral	0.49, 0.57	orange	blue
Citronellal	0.54	orange	blue
Hydroxycitronellal	0.09	orange	blue
Salicylaldehyde	0.25	purple	blue
Acrolein	0.35	red	blue
Isovaleraldehyde	0.45	orange	blue
Piperonal	0.23	pale green	blue
Anisaldehyde	0.26	olive	blue

Detection. Detection of the separated hydrazones was accomplished by spraying firstly with an ethanol solution of 0.1% *p*-nitrobenzenediazonium fluoborate until the coloured spots appeared, then with a solution of 10% alcoholic KOH to form the final blue or green anions. Alternatively, the final colours can be developed by exposure of the diazonium reagent detected spots to ammonia vapour.

Results and discussion

The R_F values and colours obtained with the spray reagents for the hydrazones of some aliphatic, aromatic and terpenoid aldehydes are shown in Table I.

Fig. 1 shows the separation of $C_1 - C_8$ aliphatic aldehyde hydrazones and Fig. 2 the separation of some aromatic and terpenoid hydrazones.

The colours formed with the diazonium reagent are stable under prolonged storage, however, the blue to green anions formed with the alcoholic KOH spray fade and revert to the colours initially formed with the diazonium reagent. The hydrazone solutions are stable for about a week when stored under refrigeration. All the hydra-

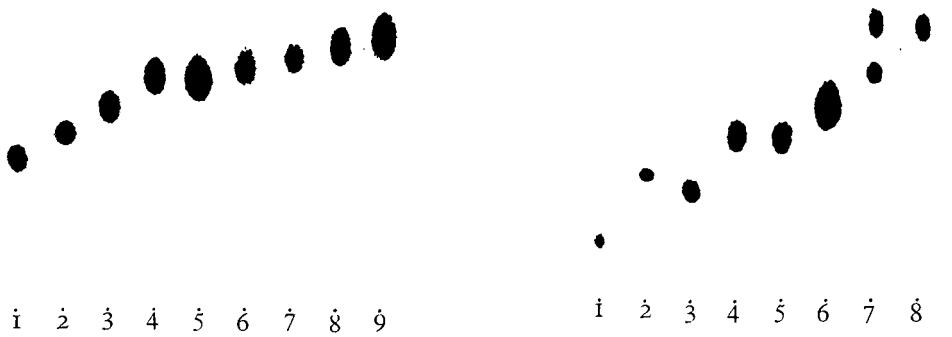

Fig. 1. Thin-layer chromatogram of C_1–C_8 aliphatic aldehyde hydrazones of 2-hydrazinobenzothiazole on Silica Gel F_{254}. Solvent: light petroleum (b.p. 30–40°)–ethyl acetate–acetic acid (88:10:2). Hydrazones of : (1) formaldehyde; (2) acetaldehyde; (3) propionaldehyde; (4) butyraldehyde; (5) isobutyraldehyde; (6) 2-methylbutyraldehyde; (7) hexyl aldehyde; (8) heptyl aldehyde; (9) octyl aldehyde.

Fig. 2. Thin-layer chromatogram of some aromatic and terpenoid aldehyde hydrazones of 2-hydrazinobenzothiazole on Silica Gel F_{254}. Solvent: light petroleum (b.p. 30–40°)–ethyl acetate–acetic acid (88:10:2). Hydrazones of: (1) hydroxycitronellal; (2) salicylaldehyde; (3) anisaldehyde; (4) *p*-tolualdehyde; (5) benzaldehyde; (6) cuminyl aldehyde; (7) citral; (8) citronellal.

zones are crystalline except those of isovaleraldehyde and citral, which are obtained as waxy solids.

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CHROM. 3954

Metabolism of xanthine alkaloids in man

Despite the world-wide occurrence of xanthine alkaloids (caffeine, theobromine and theophylline) and the consumption of relatively enormous quantities in a variety of beverages, remarkably little is known about their metabolism in man.

Urinary excretion products have been studied following the ingestion of a large dose of one particular xanthine. CORNISH AND CHRISTMAN[1] found methylated uric acids as well as 1-methyl-, 7-methyl-, and 1,7-dimethylxanthine (paraxanthine) after caffeine ingestion. SCHMIDT AND SCHOYERER[2] in similar studies detected caffeine, theobromine, paraxanthine and 1-methylxanthine in the urine of subjects treated with 300 mg caffeine orally.

It was decided to attempt the separation and identification of caffeine and its metabolites in human blood as a preliminary to further studies of caffeine metabolism in man. After many trials a two-stage system for thin-layer chromatographic separation was devised which achieved an adequate separation of the xanthines after a preliminary removal of blood lipids.

Plasma or red cells, separated from heparinized blood, was extracted twice by shaking with 3 volumes of chloroform (chloroform extracts methylxanthines but not uric acids). The chloroform extracts were clarified by centrifugation and evaporated to dryness. The extracts were then applied to plates prepared by slurrying Kieselgel G according to STAHL with phosphate buffer (pH 6.8) and spreading on glass plates to a thickness of 300 μ. The plates were used after drying and heating to 105° for 30 min. Plasma extracts (20 μl) were applied for runs of 15 cm and 2 μl of appropriate standards were applied at concentrations of 2 μg/ml in 2 N NH_3. Detection was effected by spraying with I_2/KI in ethanol (which coloured the plates brown all over) followed by 95% ethanol containing 25% conc. HCl. By this procedure caffeine was revealed as a brown spot and the dimethylxanthines as purple-blue spots against a yellow background. Spots faded rapidly and required immediate marking. The sensitivity was about 1.0 μg. Since lipids are also stained by this procedure the plates were also sprayed with 5% phosphomolybdic acid in ethanol and heated to 120° for 5 min. Lipids were revealed as blue spots.

Many solvent systems were tried for separation of caffeine and dimethylxanthines. Chloroform–ethanol (9:1) gave no separation of theobromine and paraxanthine and lipids streaked considerably over the xanthine area. Altering the ratio of the two solvents gave no improvement. Butanol–acetic acid–water (4:1:1) was much more successful. Although runs were lengthened to 3 h the separation was greatly improved. R_F values were: caffeine 0.42; theobromine 0.38; paraxanthine 0.47. Butanol–formic acid–water (33:1:7) gave very similar results.

The presence of blood lipids in the chloroform extracts at first complicated the separation of the xanthines since the lipids ran into the same areas in these solvent systems. However, the problem was resolved by the discovery that when the extracts were first run in petroleum ether (b.p. 60–80°) the lipids moved a long way (R_F approx. 0.70) while the xanthines remained on the start line. A general procedure was therefore adopted of pre-running the extracts in petroleum ether and then re-running in butanol–acetic acid–water.

This procedure has now been applied to a series of human volunteers with the following results. (1) Habitual consumers of beverages containing caffeine require about 7 days of abstention from them before caffeine completely disappears from their blood extracts. (2) In such "decaffeinated" subjects administration of 500 mg caffeine orally leads to the appearance of caffeine and paraxanthine in both red cells and plasma within 3 h. (3) Caffeine and paraxanthine then disappear from the blood extracts within 24 h.

From these results it would appear that the first step in the metabolism of caffeine in man is the removal of the 3-methyl group and the formation of 1,7-dimethylxanthine (paraxanthine). Why it takes so long to "decaffeinate" the habitual caffeine consumer as compared with the "decaffeinated" subject treated with an acute dose of caffeine is not known and further work is being undertaken.

I should like to express my gratitude for the enthusiastic collaboration of all the B.Sc. students who have taken part in this work over the past two years.

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CHROM. 3939

Note on a selective test for the detection of sulphur amino acids

Generally, detection of materials on chromatograms employs reagents that react with all members of a group or class[1]. However, the need sometimes arises, as in the study of nutritional problems of proteins, for a selective reagent to detect an individual amino acid, or a small group of acids, such as those containing sulphur.

Comparatively few selective reagents have been used for the detection of amino acids. The ninhydrin reagent has been modified[2], while sodium nitroprusside has been used for the detection of –SH containing compounds such as cysteine, and sodium cyanide for submicro amounts of cystine[3]. A chlorine–tolidine reagent[4] has been employed in studies on disulphide interchanges in proteins[5].

Incidental to other work on the detection of organo-sulphur compounds[6], it was observed that sulphur-containing amino acids responded to the test. The possibility of using it as a selective test, and of applying it to nutritional studies, suggested a more detailed study of the reaction should be made.

The principle of the test, which depends upon the reaction of the sulphur atom of the amino acid with a halogen in the presence of an aromatic amine, has been discussed previously[6], in establishing the need for the aromatic amine. Negative results were obtained with aliphatic amines, such as benzylamine, and sulphur amino acids with bromine alone do not respond. The ensuing co-oxidation results in the formation of a coloured species, and although an attempt has been made to explain the nature of this in one instance[7], its composition remains uncertain.

Experimental

Reagents. Aniline (redistilled) 3% v/v in light petroleum (b.p. 40–60°), ninhydrin 0.3% in ethanol.

Procedure. Solutions of amino acids, singly or in admixture, were applied to Whatman No. 4 paper and developed in the ascending manner using *n*-butanol–water–acetic acid (4:4:1) as solvent.

When the solvent had travelled 16–20 cm, the paper was removed, dried in a current of warm air and cut vertically into strips.

Each strip was drawn through the aniline solution and the petroleum solvent allowed to evaporate in the air. The strip was then exposed immediately to the vapours of bromine contained in an open dish, making sure the strip was thoroughly treated. Fumes of HBr were evolved at this stage. Sulphur amino acids were revealed as mauve to brown-mauve coloured spots within a few minutes.

Results and discussion

The test was applied to a short series of sulphur-containing amino acids, and for comparison, to three non-sulphur amino acids. In control tests the same series of seven compounds was chromatographed and treated with the ninhydrin reagent. Results obtained by both means of detection are shown in Table I.

Best results were obtained for cysteine and glutathione (detection limit, 10 $\mu g/cm^2$), but larger amounts of cystine (125 μg) and methionine (750 μg) were required for a positive test.

TABLE I

CHROMATOGRAPHY AND DETECTION OF AMINO ACIDS

Amino acid	*Aniline–bromine test*			*Ninhydrin test*
	Colour obtained	*Sensitivity ($\mu g/cm^2$)*	*R_F value*	*R_F value*
Glutathione	Brown-mauve	10	0.28	0.29
Cysteine	Brown-mauve	10	0.35	0.37
Cystine	Pale pink-brown	125	0.12	0.13
Methionine	Pale yellow-brown	750	0.61	0.50
Proline	No colour	—	—	0.36
Tyrosine	No colour	—	—	0.47
Leucine	No colour	—	—	0.63

The sensitivity of the test for cystine was improved by first reducing with metal and acid to cysteine. In trials, 250 μg, 125 μg, 50 μg, 25 μg, 10 μg and 5 μg were applied to the paper and chromatographed. Only the 250 μg spot could be readily detected, and the 125 μg spot with a little more difficulty. After reduction of the parent solution with zinc and dilute HCl, the same amounts of solution were again applied and chromatographed. In this case, all six spots could be readily detected by the test, and the coloured areas were now located at the position on the chromatogram appropriate for cysteine, *i.e.* closer to the solvent front.

Effects of residual acid on the paper after chromatography were overcome by double treatment of the cut out strips with the aniline solution.

The most reactive of the sulphur-containing amino acids are clearly those containing the –SH group. This is demonstrated by the positive reactions given by both cysteine and glutathione, and is reinforced by the lesser degree of sensitivity shown by cysteine containing the –S·S– group, and the comparatively insensitive reaction shown by methionine containing the thioether group.

Other amino acids, proline, tyrosine and leucine, in large amounts, did not respond to the test, thus confirming its selectivity for cysteine and glutathione, and for cystine when reduced. It appears to be a useful technique for detecting sulphur amino acids in the presence of other amino acids where overlapping R_F values are encountered (Table I).

The cyanide, iodoplatinate and iodopalladium reagents discussed by TOENNIES AND KOLB[3] are considerably more sensitive (by a factor of about 15 for cysteine). However, the tests are different in character and depend on inhibition of colour development at the site of the S-amino acid thus giving bleached spots on coloured backgrounds. By contrast in the present test colour is obtained at the site of the amino acid. The nitroprusside reagent[3] gives a brilliant red colour with cysteine but it was observed here that rapid fluctuation in the shade and intensity of colour occurs on standing.

In veterinary diagnostic work the need sometimes arises for the application of confirmatory tests to some particular biochemical investigation. Using the present test it was possible to confirm the identity of major and minor constituents of uncommon urinary calculi obtained from sheep in western New South Wales. Appli-

cation of the test to chromatograms of calculi extracts revealed the presence of cystine as a major component, with traces of methionine. The test also showed the presence of only traces of organic sulphur compounds in a calculus consisting predominantly of calcium carbonate.

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News

Meetings

XVIIth Colloquium on Protides of Biological Fluids

The XVIIth Colloquium on Protides of Biological Fluids will be held between April 30th and May 4th, 1969 in Bruges, Belgium. Correspondence should be directed to XVIIth Colloquium on Protides of Biological Fluids, P.O. Box 71, Bruges 1, Belgium.

17. Vortragstagung der Deutschen Gesellschaft für Arzneipflanzenforschung

Die 17. Vortragstagung der Deutschen Gesellschaft für Arzneipflanzenforschung e.V. findet in der Zeit vom 31. Mai bis 3. Juni 1969 in Kiel statt.

Hauptthemen:

(I) Pflanzliche Arzneistoffe aus dem Meer.

(II) Probleme der Arzneipflanzenverarbeitung (Veränderung bei Lagerung und Verarbeitung von der Frischdroge zur applizierbaren Form).

(III) Therapeutische Bedeutung des Moores.

Vortragsanmeldungen zu den Hauptthemen oder zu freigewählten Themen aus dem Gesamtgebiet der Arzneipflanzenforschung werden erbeten an Prof. Dr. O.-E. Schultz, Pharmazeutisches Institut der Universität Kiel, 23 Kiel, Gutenbergstrasse 76–78, Deutschland.

Die Vorträge zu den Hauptthemen sollen im Zusammenhang in der *Planta Medica* veröffentlicht werden.

Die Einladungen zur 17. Vortragstagung werden voraussichtlich Anfang März verschickt. Anfragen sind zu richten an den Schriftführer der Gesellschaft, Dr. R. Rangoonwala, 2 Hamburg 36, Bei den Kirchhöfen 14, Deutschland.

INTERNATIONAL SEMINAR ON GEL PERMEATION CHROMATOGRAPHY

More than 150 scientists attended the VIth International Gel Permeation Chromatography (GPC) Seminar in October, 1968 at Miami Beach. They participated in five technical sessions that bore witness to the remarkable expansion of this analytical technique.

Attendance at the seminar was 20% greater than at the last international GPC meeting. A total of 27 papers, plus two panel discussions, were presented. Plans are now under-way for the next three seminars.

The large international attendance was apprised of the wide range of applications for GPC that have developed since its introduction by JOHN C. MOORE at Dow Chemical Co. in 1964.

Typical of the widely varying applications for GPC reported at the seminar were: Analysis of commercial epoxy resins at the Bendix Corp., by Dr. FRED N. LARSEN; The molecular-weight determinations of fluids used in inertial guidance instruments (bromotrifluorethylene telomers) described by J. R. STEMNISKI of the MIT Instrumentation Laboratory; and the analysis of coal extracts at Consolidation Coal Co. reported by ROBERT J. LIMPERT AND E. L. OBERMILLER.

The seminar also indicated the increasing interest in universal methods of calibration and more mechanized means of applying correction factors to GPC results and interpreting gel permeation chromatograms.

D. D. BLY of the DuPont Experimental Station; D. J. POLLOCK AND R. F. KRATZ of Koppers Co.; A. E. HAMIELEC AND S. T. BALKE of McMaster University; G. A. FELDMAN AND W. V. SMITH of Uniroyal, Inc.; K. A. BONI AND F. A. SLIEMERS of Battelle Memoral Institute; and W. A. DARK AND J. L. WATERS of Waters Associates were among those reporting on techniques for GPC data interpretation, calibration and handling.

The VIIth International Seminar on Gel Permeation Chromatography will be held in Tokyo on March 21st and in Osaka on March 25th, 1969. In addition to the formal presentation there will be a one-hour panel discussion at the end of each day.

The VIIIth International Seminar on Gel Permeation Chromatography will be held in Monaco during October 1969, the exact dates will be available in March/April.

The IXth GPC Seminar will be held in October 1970 in the United States. Further details will be announced later.

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Paper Chromatography

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3. TECHNIQUES I (MATERIAL, SOLVENTS, DEVELOPMENT, DETECTION, QUANTITATIVE ANALYSIS)

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See also TLC section.

4. TECHNIQUES II (AUTOMATION, PREPARATIVE PROCEDURES)

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